



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/74, C07K 14/195, A61K 39/02, 39/112		A1	(11) International Publication Number: WO 98/48026
(21) International Application Number: PCT/EP97/06933			(43) International Publication Date: 29 October 1998 (29.10.98)
(22) International Filing Date: 11 December 1997 (11.12.97)			
(30) Priority Data: 97 106 503.2 18 April 1997 (18.04.97)		DE	
(71) Applicant (for all designated States except US): GESELLSCHAFT FÜR BIOTECHNOLOGISCHE FORSCHUNG MBH [DE/DE]; (GBF), Mascheroder Weg 1, D-38124 Braunschweig (DE).			(74) Agents: BOETERS, Hans, D. et al.; Boeters & Bauer, Bereiteranger 15, D-81541 München (DE).
(72) Inventors; and			(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(75) Inventors/Applicants (for US only): DARJI, Ayub [IN/DE]; Mascheroder Weg 1, D-38124 Braunschweig (DE); GUZMÁN, Carlos [IT/DE]; Mascheroder Weg 1, D-38124 Braunschweig (DE). TIMMIS, Kenneth [GB/DE]; Mascheroder Weg 1, D-38124 Braunschweig (DE). WEHLAND, Jürgen [DE/DE]; Mascheroder Weg 1, D-38124 Braunschweig (DE). WEISS, Siegfried [DE/DE]; Mascheroder Weg 1, D-38124 Braunschweig (DE). GERSTEL, Birgit [DE/DE]; Mascheroder Weg 1, D-38124 Braunschweig (DE). CHAKRABORTY, Trinad [SG/DE]; Frankfurter Strasse 107, D-35392 Gießen (DE). WACHHOLZ, Petra [DE/DE]; Mascheroder Weg 1, D-38124 Braunschweig (DE).			

(54) Title: ATTENUATED SALMONELLA STRAIN USED AS A VEHICLE FOR ORAL IMMUNIZATION

(57) Abstract

An attenuated strain of *Salmonella typhimurium* has been used as a vehicle for oral genetic immunization. Eukaryotic expression vectors containing the genes for β -galactosidase, or truncated forms of ActA and listeriolysin – two virulence factors of *Listeria monocytogenes* – that were controlled by an eukaryotic promoter have been used to transform a *S. typhimurium aroA* strain. Multiple or even single immunizations with these transformants induced a strong cytotoxic and helper T cell response as well as an excellent antibody response. Multiple immunizations with listeriolysin transformants protected the mice completely against a lethal challenge of *L. monocytogenes*. Partial protection was already observed with a single dose. ActA appeared not to be a protective antigen. The strength and the kinetics of the response suggested that the heterologous antigens were expressed within the eukaryotic host cells following transfer of plasmid DNA from the bacterial carrier strain. Transfer of plasmid DNA could be unequivocally shown *in vitro* using primary peritoneal macrophages. The demonstration of RNA splice products and expression of β -galactosidase in the presence of tetracycline – an inhibitor of bacterial protein synthesis – indicated that the gene was expressed by host cells rather than bacteria. Oral genetic immunization with *Salmonella* carriers provides a highly versatile system for antigen delivery, represents a potent system to identify candidate protective antigens for vaccination, and will permit efficacious generation of antibodies against virtually any DNA segment encoding an open reading frame.

Published

With international search report.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

ATTENUATED SALMONELLA STRAIN USED AS A VEHICLE FOR ORAL IMMUNIZATION

Introduction

The design of efficient vaccines against infectious diseases remains a major challenge in medical science. Low cost, non-invasive administration, life-long protection by single doses combined with ease of preparation, storage and transport are desirable goals to be achieved. In this respect, live attenuated bacterial carriers that express heterologous antigens are attractive vehicles for the oral delivery of vaccines. This type of delivery should result in a broad spectrum of both mucosal and systemic immune responses. Use of vaccine vectors overcomes some of the limitation of oral delivery of proteins, which usually need to be co-administered with adjuvant proteins such as cholera toxin to evoke an immune response (Brown et al., 1987; Flynn et al, 1990). In addition, administration of live replicating vectors might be advantageous over other forms of administration such as microencapsulation because of the immunomodulatory properties of cell wall components of bacteria. Finally, the natural route of entry could prove to be of benefit since many bacteria like *Salmonella* egress from the gut lumen via M cells into Peyer's Patches (Jones et al, 1994; Neutra et al, 1996; Siebers and Finley, 1996) and migrate eventually into lymph nodes and spleen, thus allowing targeting of vaccines to inductive sites of the immune system.

Genetic immunization has recently provided a promising new approach to the vaccination problem (for review see Donnelly et al., 1997). Isolated plasmid DNA - introduced into muscle or skin of the host - leads to expression of antigen in the host cells when transcription is driven by eukaryotic control elements. This has led to B and T cell stimulation and to protective responses. How these responses are generated remains still unclear. Muscle cells apparently express low levels of MHC class I but lack MHC class II and costimulatory molecules. Although, it is not known which cells function as antigen presenting cells (APC) under these circumstances, it is likely that resident dendritic cells or macrophages capture the antigen and migrate to lymph nodes and spleen to stimulate CD4⁺ and CD8⁺ T cells. Indeed antigen expressing dendritic cells have been observed after genetic immunization into the skin using a gene gun (Condon et al., 1996). It is not known whether DNA is also transferred directly into dendritic cells when plasmids are applied into muscles.

Several advantages have been observed with genetic immunization over conventional vaccination. The DNA can be detected for a considerable period of time thus acting like a depot

of antigen (Ning et al, 1993). Sequence motifs in some plasmids are immunostimulatory and can function as adjuvant (Krieg et al., 1995; Messina et al., 1991; Yamamoto et al., 1992). Co-expression of cytokines enhance the response and offer the possibility of modulating the induction of an immune response into a desired direction (Zhignan et al, 1995; Geissler et al, 1997; Kim et al, 1997). However, several obstacles need to be overcome before general applicability can be achieved.

If it would be possible to deliver plasmids for genetic immunization with an attenuated bacterial carrier, the advantages and versatilities of both systems would be combined. In addition, the natural route of administration would deliver DNA to cell types which have specifically evolved to induce immune responses. *Salmonella spp.* are particularly suited for this purpose because of the extensive knowledge on the genetics and physiology of many strains. A large body of documentation exists on their utility as heterologous antigen carriers that are capable of inducing protective immune responses (Fairwether et al., 1990; Molina et al., 1990; Newton et al., 1989; reviewed Chatfield et al, 1994; Roberts et al, 1994). Also, safe attenuated strains of *Salmonella* are available and are already in use as vaccines in animal husbandry and man (Hassan, 1996; Steinbach, 1996; Fox, 1997; Germanier and Fürer, 1975). Finally, recombinant plasmids constructed in laboratory strains of *E. coli* can be directly introduced into *Salmonellae* without further manipulations.

To sum up, we report that orally administered *S. typhimurium aroA* carrying plasmids encoding β -galactosidase (β -gal) of *Escherichia coli*, or truncated forms of ActA or listeriolysin of *Listeria monocytogenes* under the control of an eukaryotic promoter induce an efficient humoral and cellular immune response. The strength and kinetics of the response is only compatible with the interpretation of a transfer of the expression plasmid from the *Salmonella* carrier to the nucleus of APC of the host. β -galactosidase activity was detectable even five weeks after administration of the oral vaccine. In addition, *in vitro* experiments with mouse primary macrophages demonstrated an efficient transfer of plasmid DNA from attenuated bacteria into the nucleus of phagocytic host cells.

Results

To achieve genetic immunization with a live attenuated bacterial carrier three plasmids were used which are based on the commercially available plasmid pCMV β . This plasmid contains the structural gene of β -gal under the control of the human CMV immediate early promoter and includes a splice donor and two splice acceptor sites in between the promotor and the structural gene. For studies examining the efficiency of the immune response against pathogens the β -gal gene was replaced by genes encoding two virulence factors of *Listeria monocytogenes*. A truncated gene encoding a non-hemolytic variant of listeriolysin (pCMVhly) from amino acid positions 26 to 482 and a truncated variant of the structural gene of the membrane protein ActA (pCMVactA) encoding amino acid 31 - 613 were used. *S. typhimurium aroA* strain SL7207 was transformed with these three plasmids and groups of mice were orally immunized by feeding 10^8 organisms to each mouse per immunization. This dose was found to be optimal (data not shown). The mice did not show any overt signs of illness using this immunization schedule.

*Induction of a strong T cell response by immunization with *Salmonellae* carrying eukaryotic expression vectors*

The working hypothesis of these experiments is that orally administered *S. typhimurium aroA* would result in uptake of the bacteria by macrophages and/or dendritic cells, with concomitant activation by the endotoxin of the bacteria. Following a few rounds of bacterial division the intracellular bacteria would die because of their inability to synthesize essential aromatic amino acids. During this process plasmids would be released and transferred into the cytosol and the nucleus of the infected cells. Eventually, the encoded genes will be expressed by host APC.

The first prediction of this hypothesis is the induction of a strong cytotoxic response of CD8 T cells, since antigen would be expressed in the cytosol, the cellular compartment responsible for MHC class I presentation. To this end, two kinds of experiments were performed. Mice were either infected orally once with recombinant *Salmonellae* and their cytotoxic T cell responses were followed for several weeks by testing their spleen cells directly *ex vivo* (data not shown) or after one restimulation *in vitro*. Alternatively, mice were orally immunized four times at two weeks intervals and the course of the cytotoxic response was examined. Fig. 1 demonstrates

that a strong and specific CD8 T cell response can be elicited with orally administered *Salmonella* carrying eukaryotic expression plasmids. Mice immunized with the truncated gene of listeriolysin elicited only a response towards targets sensitized with the immunodominant peptide comprising AA91-99 of listeriolysin (LLO) and not against targets sensitized with soluble hen egg lysozyme (HEL) or a control peptide (Fig. 1A). Similarly, spleen cells from mice immunized with *Salmonella* carrying the ActA expression plasmid could only respond to ActA (Fig. 1D). To reveal the cytotoxic response against ActA, we exploited the pore-forming activity of listeriolysin. This activity of listeriolysin allows the introduction of soluble passenger proteins into the cytosol of target cells (Darji et al., 1995a; Darji et al., 1997). Stimulators and target cells were therefore sensitized with a mixture of soluble ActA and LLO. A specific response was observed only when the combination of ActA and LLO was used. No response was found when LLO alone was tested. These responses were specific for the plasmid encoded antigen during the whole time period indicated in Fig. 1 panels B & C and E & F and were also observed when the response of mice immunized with *Salmonella* harboring the β -gal control plasmid was studied (data not shown).

The kinetic of the responses indicated that even a single dose elicited a strong cytotoxic T cell response which peaked 5 weeks after immunization and then slowly declined (Fig. 1C and F). On the other hand, the response was still rising even at the end of the observation period, i.e. 5 weeks after the last challenge in mice that had received four immunizing doses (Fig. 1B and E). Thus, a strong cytotoxic response was observed when using *Salmonella* as potential vehicle for genetic immunization.

Genetic immunization usually also evokes a CD4 helper T cell response (Donnelly et al., 1997). Therefore, cells from spleen and mesenteric lymph nodes of the same mice used above were tested for their proliferative response against soluble proteins. This type of response is mainly due to presentation of antigen via MHC class II molecules and carried out by CD4 T cells. As shown in Fig. 2, a strong and specific helper T cell response, in parallel to the cytotoxic response is observed when eukaryotic expression plasmids carried by *Salmonellae* were used for immunization (Fig. 2A and D). As with the CD8 response, a single dose was sufficient for a good response which was still increasing at the end of the observation period regardless of whether listeriolysin or ActA was used as antigen (Fig. 2C and F). Four consecutive immunizations however, resulted in an even stronger response which appeared long lasting

since the response apparently was still increasing five weeks after the last challenge (Fig. 2B and E). Similar results were obtained with *Salmonella* carrying the control plasmid expressing β -gal (data not shown). Analysis of the supernatants of the *in vitro* cultures revealed production of IFN γ by these T cells. No IL-4 could be found, suggesting that such an immunization scheme is mainly inducing a TH1 or inflammatory type of T helper response.

*Induction of specific antibodies by immunization with *Salmonellae* carrying eukaryotic expression vectors*

Pooled sera of the groups of mice used above were tested for the presence of specific antibodies. Clearly, in addition to a cytotoxic and helper T cell response, immunization with *Salmonellae* carrying eukaryotic expression plasmids induced strong and specific antibody responses as revealed by ELISA (Fig. 3A and B) or immunoblot (data not shown). Again a single immunization was sufficient for a good response which peaked four weeks after the administration of the bacteria and then declined in the same way as seen for cytotoxic response (Fig. 3A and B). Four immunizations did not increase the antibody titer significantly but probably induced a longer lasting response since a plateau of antibody titer was not reached even at the end of the observation period (Fig. 3A and B).

The analysis of the subclass distribution of individual mice at week 11 indicated a high concentration of IgG2a while the concentration of IgG2b and IgG3 was negligible (Fig. 3 C and D). This is in agreement with the finding that only IFN γ and no IL-4 could be detected in the supernatant of the restimulated T helper cells. However, IgG1 was also observed at high concentrations in the immune sera. This subclass is found when TH2 helper cells are taking part in the immune response, indicating that under our experimental conditions TH2 cells might also be induced but were not revealed in the *in vitro* T cell assay. In addition, IgA antibodies were evoked by this immunization schedule (not shown).

Taken together the results presented in Fig. 1-3 show that immunization with *S. typhimurium* *aroA* carrying eukaryotic expression vectors can evoke responses in all three specific effector compartments of the immune system, namely, cytotoxic CD8 T cell, CD4 T cells and antibodies. The response in the T helper compartment was strongly biased towards a TH1 or inflammatory T helper response.

*Protection against lethal doses of *L. monocytogenes**

The strong response observed, in particular that of cytotoxic T cells, suggested that mice immunized in such a way should be protected from a lethal dose of *L. monocytogenes*. Therefore, 90 days after the first immunization or 48 days after the fourth immunization - where applicable - mice were challenged *i. v.* with a dose of bacteria corresponding to 10 x LD₅₀. Fig. 4 shows that animals which were immunized four times consecutively with *Salmonellae* harboring an eukaryotic expression vector that encodes truncated LLO were completely protected (Fig. 4A). Animals that had received a single vaccination only were partially but significantly protected since at the time of termination of that experiment 60% of the animals were still alive. All animals that were immunized with *Salmonellae* that carried the β -gal control plasmid were not protected and died within four days. Surprisingly, immunizations with *Salmonellae* carrying the ActA expression plasmid did not result in protection, although strong cytotoxic and helper T cell responses could be demonstrated in mice from the same group indicating that the immunization had been successfull (data not shown). Thus, the listerial membrane protein ActA is not a protective antigen.

*Evidence for transfer of the expression plasmid from the carrier *Salmonellae* to host cells in vivo*

We were concerned that a weak activity of the eukaryotic promoter in the bacteria or a cryptic prokaryotic promoter in the plasmid could result in expression of the antigens in the bacterial carrier thus eliciting the potent immune response. In fact, the recombinant *Salmonellae* harboring the pCMV β exhibited low β -gal activity (2.5 U) compared to the parental strain. To rule out any possibility, we immunized mice with a recombinant *Salmonella* strain that produced more than 100 fold higher levels (334 U) of β -gal enzymatic activity. A single vaccinating dose using these bacteria did not elicit any measurable T cell or antibody response (Fig. 5A-C). Repeated vaccination, however, resulted in a weak cytotoxic T cell response detectable after *in vitro* restimulation, although, it barely reached the strength of the response observed using a single immunization with *Salmonellae* harboring the eukaryotic expression plasmid of β -gal (Fig. 5A). Neither a CD4 T cell nor an antibody response was observed even after repeated oral immunization with *Salmonellae* constitutively expressing β -gal (Fig.5B and C).

7

As a result of the *aroA* mutation bacteria appear to die very quickly since live bacteria could never be demonstrated after immunization at various time points examined. Nevertheless, even at five weeks following oral administration of *Salmonellae* harboring the eukaryotic β -gal expression plasmid, enzymatic activity of β -gal could be detected in adherent cells - most likely macrophages - from the spleen of these mice suggesting plasmid transfer to the eucaryotic cell (data not shown). To further corroborate this observation we injected *Salmonellae* carrying the pCMV β vector into the peritoneum of mice and harvested the peritoneal exudate cells after 1 hour. Cells were then cultured overnight in the presence of tetracycline to inhibit bacterial protein synthesis and finally stained for β -gal activity. Enzymatic activity of β -gal was observed in a large number of macrophage like cells. The staining was diffuse and clearly not restricted to the endocytic vesicles in which *Salmonella* usually reside. This suggests that plasmid DNA was transferred from dying *Salmonellae* to host cells and had occurred at a high frequency.

DNA transfer from S. typhimurium aroA to mammalian host cells in vitro

To obtain direct evidence that DNA transfer from the bacterial carrier to the mouse macrophages can take place, primary peritoneal macrophages were infected with *Salmonellae* harboring the β -gal expression plasmid (pCMV β). After infection for one hour, gentamicin was added to kill remaining extracellular bacteria. Four hours later tetracycline was added to kill resident intracellular bacteria. After overnight incubation, cells were stained for β -gal activity. In up to 30 % of the adherent, macrophage-like cells, enzymatic activity could be demonstrated even in the continuous presence of tetracycline which blocks bacterial protein synthesis (Fig. 6).

To show that β -galactosidase was produced by the host cell, and not by the bacteria, two type of experiments were performed. Firstly, adherent peritoneal cells were infected and treated as described above. After overnight incubation RNA was extracted. If the plasmid had indeed been transferred and transcribed in the nucleus of the host cell, RNA splice products derived from the splice donor and acceptor sites within the vector should be demonstratable. By RT-PCR with a primer pair that hybridises to sequences on either side of the small intron, a PCR product could be observed which corresponded to one of the expected splice products (Fig. 7A). The identity of this product was confirmed by DNA sequencing (data not shown).

Secondly, biosynthetic labelling of proteins in the presence of tetracycline should only allow translation of mRNA produced by the eukaryotic host cells. Adherent peritoneal cells were infected as described and were pulsed for 30 min with ^{35}S -methionine after 4, 24 or 48 hours in the absence or presence of tetracycline. At four hours no β -gal could be observed by immunoprecipitation, even in the absence of tetracycline where bacterial products should have been labelled (Fig. 7B). Thus, transfer of plasmid DNA and eukaryotic expression had not taken place yet. However, β -gal could be immunoprecipitated following a 24 hour or 48 hour incubation period even when tetracycline was continuously present during both the incubation and labelling period. Preincubation of the anti- β -gal antibody with an excess of unlabeled β -gal demonstrated the specificity of the immunoprecipitation (Fig. 7B/lane 10). This clearly indicates that the β -gal precipitated was produced by the infected mammalian host cell itself and not by the bacterium which had originally carried the expression plasmid. Thus, a transfer of the plasmid from *Salmonellae* to the host cell must have taken place.

Discussion

The transfer of eukaryotic expression plasmids from attenuated enteric bacteria into the nucleus of host cells has recently been demonstrated. While this work was in progress it was reported that auxotrophic mutants of *Shigella* and *E. coli* that express the invasin of *Shigella* can carry eukaryotic expression plasmids into host cells (Sizemore et al., 1995; Courvalin et al., 1995). Given that both bacteria are capable of escape from the phagolysosome into the cytosol of the host cell, it follows that lysis of bacteria in this compartment would allow transfer of the released plasmid into the nucleus. Transfer of plasmid from intracellular pathogens such as *Salmonella* would be harder to imagine as these bacteria are generally retained within vacuoles of the infected host cell. Indeed, only a „low efficiency“ of plasmid transfer into a macrophage cell line using attenuated *Salmonella* had been reported (Sizemore et al., 1995). Our initial experiments using several macrophage cell lines had also indicated that this was indeed the case (data not shown).

However, the kinetic and strength of the immune response after administering *Salmonella* carrying eukaryotic expression vectors suggested that a plasmid transfer might have taken place *in vivo*. We therefore decided to investigate primary macrophages isolated from the peritoneum of mice. Using these cells we could clearly demonstrate a transfer of an eukaryotic expression plasmid vector into host cells. A pathway that permits transfer of proteins from endocytic vesicles into the cytosol of some cell types including macrophages has been described (Reis de Sousa and Germain, 1995; Norbury et al., 1995). Whether such a pathway could also be responsible for the transfer of nucleic acids observed here remains to be studied. The fact that plasmid transfer with *Salmonella* was only observed with primary macrophages and not with cell lines suggests the presence of a transport pathway which is only operating efficiently in primary cells.

Evidence for a transfer of plasmid DNA from *Salmonella* to the host cell *in vitro* is compelling. Splicing of RNA and protein synthesis in the presence of tetracycline are both only possible if the gene is expressed by the eukaryotic host cell. Evidence that transfer of the expression vector *in vivo* is responsible for induction of the strong immune response observed, also was obtained. Enzymatic activity of β -gal could be observed five weeks after the last challenge in a few adherent spleen cells. However, viable *Salmonella* could not be detected even when tested one

week after the last infection, thus, arguing that β -gal expression cannot be due to residual surviving *Salmonella*. Nevertheless, it is intriguing how such antigen expressing cells can coexist in the presence of specific cytotoxic T cells.

Strong cytotoxic and protective responses have only been reported with *Salmonella* that secrete the antigens. No comparable responses have been described using *Salmonella* that constitutively express nonsecreted heterologous proteins (Hess et al., 1996). High doses of recombinant bacteria that express intracellular protein were required to induce CD8 T cells (Turner et al., 1993). Although induction of specific antibodies have been described under some experimental conditions (Guzman et al., 1991; Walker et al., 1992) no antibody response was observed under the circumstances described above (Turner et al., 1993). This was confirmed by our own results (Fig. 5). We therefore find it highly unlikely that the strong responses of cytotoxic and helper T cells as well as the specific antibody production is the result of a fortuitous expression of the antigens in the *Salmonella* carrier.

The strength of the immune response observed especially after a single dose of immunization indicates that transfer of DNA by bacterial carrier is probably superior to a direct application of isolated plasmid DNA into skin or muscles. This suggests that by using the natural port of entry of a pathogen, the expression vector is transferred into cell types that have evolved to efficiently induce an immune response. It is likely that the *Salmonella* carrier is taken up by macrophages and dendritic cells. Whether, macrophages play a role during stimulation of naive T cells against bacteria is not clear, but dendritic cells are known to be highly efficient in priming resting T cells. Since the antigen is expressed in the cytosol of these cells a strong cytotoxic T cell response is to be expected.

The induction of an additionally strong helper and antibody response is puzzling and can only be speculated upon. Some cytosolic proteins can efficiently be presented by MHC class II molecules (Brooks and McCluskey 1993). However, it would be a very fortunate coincidence if all three proteins used in the present study display this property. In any case, it could not explain the antibody responses that we observed. It is more likely that APC expressing the antigen are lysed by specific cytotoxic cells and dying antigen containing cells or free antigen is taken up by neighbouring APC and presented via MHC class II molecules. The generated humoral response could be explained in a similar way.

//

In summary, oral genetic immunization using attenuated *Salmonellae* as carrier could work as schematically depicted in Fig. 8. *Salmonella* enter the host via M cells in the intestine. The bacteria are taken up in the dome areas by phagocytic cells such as macrophages and dendritic cells. These cells are activated by the pathogen and start to differentiate and probably to migrate into lymph nodes and spleen. During this time period the bacteria die due to their attenuating mutation and liberate the plasmid-based eukaryotic expression vectors. The plasmids are then transferred into the cytosol either via a specific transport system or by endosomal leakage. Finally, the vector enters the nucleus and is transcribed, thus, leading to antigen expression in the cytosol of the host cells. Specific cytotoxic T cells are induced by these activated APC which lyse antigen expressing cells. Free antigen or dying cells can be taken up by other APC, which now in turn can stimulate helper cells. Free antigen would also be responsible for the induction of an antibody response. In addition, bacterial endotoxin and DNA sequence motifs of the vector could also function as adjuvant and could contribute to the strength of the responses observed.

The helper T cell response induced with this type of genetic immunization seemed strongly biased to the TH1 type as indicated by IFNg production of restimulated T cells *in vitro* and the high titer of IgG2a in the humoral response (Mosmann and Coffman, 1989). This is not unexpected since bacteria usually induce inflammatory types of response. For many vaccination strategies it is desirable to induce an TH1 response for protection against the particular pathogen, e. g. strains of mice which respond with TH2 cells against *Leishmania major* do not clear the parasite and are not protected while mice which mount a TH1 response are resistant (Sher and Coffman, 1992). On the other hand, induction of TH2 type of responses or the conversion of a TH1 response into a TH2 response has been shown to be advantageous in inflammatory autoimmune diseases (Tian et al., 1996). Similarly, infections by nematodes might also require a TH2 response (Sher and Coffman, 1992). Since the bacteria are only being used as a vehicle in transferring the expression plasmids and therefore play only a secondary role it should be possible to manipulate the TH1 response. The induction of specific IgG1 suggests the presence of a TH2 component during the helper response that might be augmentable. Co-expression of the antigen together with certain cytokines or costimulatory molecules or alternatively using antisense strategies to suppress costimulatory molecules should make it possible to drive the responses more towards TH2.

12

Two well characterized virulence factors were tested as antigens for protection against a lethal challenge with *L. monocytogenes*. Listeriolysin has been shown before to induce protection (Harty and Bevan, 1992; Hess et al., 1996). This was also true under our experimental conditions. Interestingly, even a single dose of *Salmonellae* harboring the eukaryotic listeriolysin expression plasmid was sufficient to afford protection to 60 % of the mice. On the other hand, ActA did not serve as protective antigen. The membrane protein ActA obviously is not available to the presentation mechanisms as long as the bacteria are alive. This raises the question as to whether membrane proteins of bacteria in general are not protective or whether ActA is a special case. Extensive phosphorylation of the ActA protein by host kinases following infection may affect its ability to be processed. Nevertheless, the role of bacterial surface-bound proteins in protection can now easily be addressed using the *Salmonellae* system for genetic vaccination.

The induction of a strong and specific antibody response which can be measured in ELISA and by immunoblot revealed additional benefits derived from the type of immunization described here. Thus, to raise specific polyclonal and possibly also monoclonal antibodies, any open reading frame can be inserted into an expression plasmid and used for immunization. This will facilitate the characterization of gene products where only sequence information is available.

In conclusion: using attenuated *Salmonella* which carry eukaryotic expression vectors, genetic immunization can be achieved by oral administration of the carrier. The stimulation of cytotoxic and helper T cells as well as the induction of a strong antibody response provides a very versatile system for new immunization strategies. The strength of this approach also draws on the development of newer more rationally attenuated *Salmonellae* strains as well as technical advances in providing conditional and targeted eukaryotic expression by the infected host cell. The possibility of genetic immunization with DNA fragments containing open reading frames will allow to define the function of new gene products, provide novel serological reagents, and permit delineation and assess efficacies of protective antigens in vaccination protocols.

Now the invention is described in greater detail based on figures and experimental data. ¹³

Legends to Figures:

Fig. 1 Induction of cytotoxic T cells in mice orally immunized with 10^8 *S. typhimurium aroA* carrying eukaryotic expression plasmids which encode listeriolysin or ActA. Mice were immunized either four times with two week intervals (A, B, D, E) or once (C; F) with *Salmonella* carrying pCMVhly (A-C) or pCMVActA (D-F) and spleen cells were restimulated once *in vitro* with a synthetic peptide comprising AA91-99 of listeriolysin (A-C) or with a mixture of purified ActA and hemolytically active listeriolysin which results in the class I presentation of ActA due to the pore-forming activity of listeriolysin (Darji et al., 1995a; Darji et al., 1997). Restimulated T cells were tested with radiolabelled P815 target cells at an effector to target ratio of 10:1. A: Specificity of the anti-listeriolysin cytotoxic response. Target cells were sensitized with henegglysozyme (HEL), peptide AA 91-99 of listeriolysin (pLLO) or control peptide of nucleoprotein of influenza virus (pNP). Displayed is the experiment with spleen cells from week 5 shown in panel B. Similar specificity was observed at all other time points. B: Kinetic of the cytotoxic response of mice immunized four times with pCMVhly. The arrows indicate the booster immunizations. C: Kinetic of the cytotoxic response of mice immunized once with pCMVhly. D: Specificity of the anti-ActA cytotoxic response. Target cells were sensitized with a mixture of ActA and listeriolysin (ActA + LLO), HEL and listeriolysin (HEL + LLO) or listeriolysin alone (LLO). Displayed is the experiment with restimulated spleen cells from week 5 shown in panel E. Similar specificity was observed at other time points and including other synthetic peptides of various sources. E: Kinetic of the cytotoxic T cell response in mice immunized four times pCMVActA. Arrows indicate booster immunizations. F: Kinetic of the cytotoxic T cell response in mice immunized once with pCMVActA. The specificity of the cytotoxic response was further assessed by testing the spleen cells of mice immunized in a similar way with pCMV β (β - gal) on target cells sensitized with pLLO, ActA plus listeriolysin or a β - gal expressing transfectant of P815 (data not shown). Similarly, a specific cytotoxic T cell response was observed against β -gal., but the kinetic was not followed as systematically as for the two other antigens.

Fig. 2 Induction of helper T cells in mice orally immunized with 10^8 *S. typhimurium aroA* carrying eukaryotic expression plasmids which encode listeriolysin or ActA. Spleen (SPC) and lymphnode cells (LNC) from the same mice tested for cytotoxic T cell responses displayed in

Fig. 1 were tested for T helper responses. Mice were immunized either four times (A, B, D, E) or once (C; F) with *Salmonella* carrying pCMVhly (A-C) or pCMVActA (D, F) and restimulated *in vitro*. After two days proliferation was tested by incorporation of 3 H-Thymidine. A: Specificity of the proliferative response of spleen cells from mice immunized with pCMVhly. T cells tested were the same as those displayed in panel B at week 11. Similar results were obtained at other time points. B: Kinetic of the proliferative response of spleen and lymph node cells from mice immunized four times with pCMVhly. Arrows indicate the booster immunizations. C: Kinetic of proliferative response of spleen and lymph node cells from mice immunized once with pCMVhly. D: Specificity of proliferative response of spleen cells from mice immunized four times with pCMVActA. T cells tested were the same as those displayed in panel D at week 11. Similar results were obtained at other time points. E: Kinetic of the proliferative response of spleen and lymph node cells immunized four times with pCMVActA. Arrows indicate booster immunizations. F: Kinetic of the proliferative response of spleen and lymph node cells from mice immunized once with pCMVActA. Similarly, spleen and lymph node cells from mice immunized with pCMV β (β - gal) never reacted with either listeriolysin or ActA but could respond to restimulation with β - gal (data not shown).

Fig. 3 Kinetics and subclass distribution of specific serum IgG from mice orally immunized with *S. typhimurium* aroA carrying eukaryotic expression plasmids which encode listeriolysin, ActA or β - gal. Sera from the same mice tested for cytotoxic and proliferative T cell responses displayed in Fig. 1 and 2 were used and assayed in specific ELISA's. Mice were immunized four times (A) or once (B) with pCMVhly, pCMVActA or pCMV β respectively, and pooled sera were tested for antigen specific serum IgG. To assess specificity all sera were tested on all three antigens. Reactivity was only observed against the immunizing antigen (data not shown). Identical results were obtained by immunoblotting using the same antigens (data not shown). The subclass distribution 11 weeks after the first immunization was determined from the sera of individual mice immunized four times (closed symbols) or once (open symbols) with either pCMVhly (C) or pCMVActA (D).

Fig. 4 Oral immunization with *S. typhimurium* aroA carrying the eukaryotic expression plasmid which encodes listeriolysin induces a protective immune response, whereas immunization with bacteria carrying the expression plasmid for ActA is not protective. Groups of six mice were

15

immunized four times with two week intervals (A) or only once (B) with *Salmonella* carrying pCMVhly, pCMVActA or pCMV β and challenged with a lethal dose of 5×10^4 *L. monocytogenes* EGD ($10 \times LD_{50}$) intravenously. Mice that had been immunized only once with pCMVhly became moribund after two days. However, four of them recovered and survived in good condition until the experiment was terminated two weeks later.

Fig. 5 Comparison of orally induced immune responses elicited with *Salmonella* harboring prokaryotic or eukaryotic expression plasmids for β - gal. Mice were immunized with *Salmonella* harboring either the eukaryotic expression plasmid pCMV β or the plasmid pAH97 that constitutively expressed β - gal from the Pr and Ps promotor of *XylS* of *Pseudomonas putida*. Bacteria harboring the eukaryotic vector were administered orally once (●), whereas bacteria expressing β - gal under the control of the prokaryotic promotor were administered either once (◆) or four times with two week intervals (▼). The arrows indicate the time of booster immunizations. A. Cytotoxic response of restimulated spleen cells tested at an effector to target ratio of 10:1. The β - gal expressing transfectant P13.1 was used as target in the JAM assay. B. Proliferative helper T cell response of spleen cells with isolated β -gal as antigen. C. Antibody response against β - gal from pooled sera measured by ELISA. Data displayed in A-C were obtained with cells or sera from the same mice. All assays were performed as described in figures 1-3.

Fig. 6 Expression of enzymatic β -gal activity in peritoneal exudate cells after infection with *S. typhimurium aroA* that harbor an eukaryotic expression plasmid for β - gal. Freshly isolated peritoneal exudate cells (PECs) were allowed to adhere for two hours and infected at a MOI of 10 for 15 min with *Salmonella* bearing pCMV β in antibiotic free medium. Following a wash and addition of gentamycin to kill bacteria which remained outside of the cells, incubation was continued for 3-4 h at 37 °C. Medium was then supplemented with tetracycline to kill the bacteria by blocking their protein synthesis. After additional 24 h at 37 °C cells were washed with PBS, dried, fixed with acetone/methanol and incubated overnight with the X-gal substrate. Sometimes, expression of β - gal activity in up to 30 % of the adherent cell population was observed. Only macrophage-like cells expressed enzymatic activity. The small cells found in the displayed cultures most likely represent nonadherent lymphocytes which were not removed in this particular experiment. Tetracycline remained in the medium through the whole experiment. Staining the cells already after 4 h did not reveal any enzymatic activity.

16

Fig. 7 The eukaryotic host cells transcribe and translate β -gal derived from *S. typhimurium* *aroA* harboring the expression plasmid. A : RNA derived from PEC's 24 h after infection with *Salmonella* carrying pCMV β was analysed by RT-PCR. A primer pair that borders the splice donor and acceptors sites downstream of the promotor was used. In lane 2 a band of 196 bp (indicated by the arrow) could be detected. DNA sequencing identified this fragment as a splice product. The stronger 227 bp long fragment seen in this lane is either due to carry over of DNA into the RNA preparation or due to inefficient splicing. Lane 1 shows the untreated macrophage control and the lane marked M contains the molecular size marker used. Since the signal of the splice product was extremely weak we inverted the colours from white to black. This resulted in an increase of contrast and allowed the visualization of the splice product on the graph. B: PEC's were infected with pCMV β carrying *Salmonella* as described and after incubation at various length of time biosynthetic labelling was performed in the presence or absence of tetracycline followed by immunoprecipitation with β - gal specific monoclonal antibodies. Controls: (1) BHK cells; (2) BHK cells transfected with β -gal (positive control). Infected PEC's: (3) incubated four hours *post infection* (p.i.), without tetracycline; (4) incubated four hours p. i., with tetracycline during labelling; (5) incubated four hours p.i., with tetracycline during incubation and labelling; (6) incubated 24 h p.i., without tetracycline; (7) incubated 24 hours p.i., with tetracycline during labelling; (8) incubated 24 h p.i., with tetracycline during incubation and labelling (9) incubated 48 h p.i., without tetracycline; (10) incubated 48 h p.i., with tetracycline during labelling, a 100-fold excess of β -gal over the precipitating antibody was added to the lysate before immunoprecipitation; (11) incubation of 48 h p.i., with tetracycline during labelling; (12) incubation 48 h p.i., with tetracycline during incubation and labelling. No specific band was observed after 4 h of incubation under any conditions. However, after allowing 24 h or more for a DNA transfer and expression to occur, a specific band for β -gal - indicated by the arrow - can be observed.

Fig. 8 Schematic representation of the sequence of events that might occur *in vivo* after oral genetic immunization with attenuated *S. typhimurium* *aroA*.

17

Experimental procedures

Mice

Female BALB/c (H-2^d) mice 6-8 weeks old, were obtained from Harlan Winkelmann (Borchem, Germany).

Media, reagents and antigens

RPMI (Gibco), supplemented with 10 % fetal bovine serum, where required, was used as culture medium for eukaryotic cells and all functional assays were performed in this medium. Solid and liquid Luria Bertani medium (LB, Sambrook) was used for growing *E. coli* and *S. typhimurium* strains. Brain heart infusion broth or agar (BHI; Difco, Detroit, USA) was used for growing *L. monocytogenes* EGD. Media were supplemented, where required with 100 µg/ml of ampicillin. Concanavalin-A (con-A), hen egg lysozyme (HEL), tetracycline, β-galactosidase of *E. coli*, potassium ferrocyanide and potassium ferricyanide were purchased from Sigma (Sigma, St. Louis, USA), listeriolysin was purified as described (Darji et al., 1995b). Soluble ActA protein (AA 31-505) was purified from supernatants of recombinant *L. monocytogenes* (Gerstel et al. to be published).

Bacterial strains and plasmids

The *E. coli* strain XL1-Blue (Stratagene, Heidelberg, Germany) was used as a host during the cloning experiments and to propagate plasmids. The auxotrophic *S. typhimurium* aroA strain SL7207 (*S. typhimurium* 2337-65 derivative hisG46, DEL407 [aroA::Tn10{Tc-s}]), kindly provided by Dr. B. A. D. Stocker, Stanford, CA, USA, was used as a carrier for the *in vivo* studies. The hemolytic *L. monocytogenes* strain EGD (serotype 1/2a; Chakraborty et al., 1992) was used for *in vivo* protection assays and preparation of genomic DNA. The DNA was used as template for the PCR amplification of *actA* and *hly* genes. The eukaryotic expression vector pCMVβ (Clontech, Palo Alto, USA) containing β-gal of *E. coli* was used for the cloning by replacing β-gal with the amplification products containing a truncated variant of *actA* or of *hly*. For expression of β-gal in *Salmonellae* the plasmid pAH97 was used (Holtel et al., 1992). It contains the Pr and Ps promotor of the *Xyl S* gene of *Pseudomonas putida* and results in constitutive expression of β-gal (384 U) in the *S. typhimurium* aro A strain. Bacterial cultures were grown at 37°C and aerated by shaking at 200 r.p.m.

Recombinant DNA techniques

DNA preparation, genetic manipulations and PCR were carried out according to standard protocols (Sambrook et al., 1982), and plasmid DNA transformation of bacterial cells was performed as described by Hannahan or by electroporation (O'Callaghan and Charbit, 1990). DNA sequencing was performed using a Taq Dye Deoxy terminator cycle sequencing system (Applied Biosystems) and analyzed on an Applied Biosystems 373A automated DNA sequencer.

Cloning of actA and hly into the eukaryotic expression vector pCMVb

For the construction of the eukaryotic expression vector pCMVActA, a 1.8 kb fragment encoding AA 31 to 613 of a ActA polypeptide without the membrane anchor (Domann et al., 1992) was amplified by PCR using the forward and reverse primers:

5'-ATAAGAATGCGGCCGCCATGGCGACAGATAGCGAAGATTCTAGTC-3' and
5'-ATAAGAATGCGGCCGCTTACGTCGTATGGTTCCCTGGTTCTC-3'; and genomic DNA from *L. monocytogenes* strain EGD as template. In a similar way recombinant plasmid pCMVhly was constructed. A 1.4 kb fragment encoding a non hemolytic variant comprising amino acids 26 to 482 of *hly* and deleting the peptide essential for hemolytic activity (Mengaud et al., 1988) was amplified using the forward and reverse primers:

5'-ATAAGAATGCGGCCGCCATGGATGCATCTGCATTCAATAAGAAAATTC-3' and
5'-ATAAGAATGCGGCCGCTTATTAGCGTAAACATTAATATTCTCGCG-3'. PCR primers were designed in such a way that the resulting fragments contain *Not*I flanking restriction sites (underlined) and start and stop codons were introduced (ATG and TTA in bold). The PCR fragments were digested with *Not*I and ligated with *Not*I-digested pCMV β , thereby generating pCMVActA and pCMVhly, respectively. The coding region for β -gal is deleted from pCMVb by the *Not*I digestion. The DNA sequence of the inserted PCR fragments were verified by Taq Dye Deoxy terminator cycle sequencing.

Immunization and challenge

For immunization, groups of 5-10 female BALB/c mice were fed with 30 ml of 10% sodium bicarbonate buffer containing 10^8 recombinant *S. typhimurium* *aroA* strain harboring one of the eukaryotic expression vectors pCMV β , pCMVhly or pCMVActA or the prokaryotic β - gal expression plasmid pAH97 (Holtel et al., 1992). Mice received either a single immunization or four immunizations at 14 days intervals. Serum samples from both groups of mice were

obtained on day -1, 7, 21, 35 and 63 and were stored at -20°C until used in enzyme-linked immunosorbent assay (ELISA) or immunoblot. Mice of each group were sacrificed at weeks 3, 5, 7 and 11 after the first immunization and tested for T cell responses. For protection studies, immunized mice were challenged *i.v.* on day 90 (one and half month following the last boost of mice receiving multiple immunizations) with a lethal dose of 5×10^4 *L. monocytogenes* EGD. Survival of mice was followed until day 14 post-challenge.

CTL assay

For the determination of induction of cytotoxic T cells the JAM assay was performed (Matzinger P., 1991). Briefly, 3×10^5 target cells were incubated for 4 h with 5 μ Ci 3 H-thymidine (Amersham), washed and co-cultured with the spleen cells isolated from mice immunized with *S. typhimurium* *aroA* strains, harboring the eukaryotic or prokaryotic expression vectors at different effector to target ratios. Spleen cells were either assayed straight *ex vivo* or after *in vitro* restimulation for 5 days. To test for LLO specific cytotoxic T cells, P815 target cells were sensitized with 1 μ g/ml of LLO peptide AA 91-99 (Parmer et al., 1991). The ActA specific cytotoxicity was revealed by sensitizing the radiolabeled P815 cells with a mixture of 1 μ g/ml purified hemolytically active LLO and 1 μ g/ml of purified ActA protein for 30 min at RT. We have shown previously that it is possible to sensitize target cells very efficiently *in vitro* with soluble proteins by using the pore-forming activity of LLO. Target cells sensitized with LLO only were not lysed when T cells from mice that were immunized with *Salmonella* carrying the ActA expression plasmid were tested. This indicates that the assay is specific for ActA when mice were immunized with *Salmonellae* harboring ActA expression plasmids. To measure the β - gal specific cytotoxicity, P13.1 - a P815 derivative transfected with the β - gal gene - was used as target cells (Rammensee et al., 1989). Mixtures of effector and target cells were incubated for 4-5 h at 37°, then plates were harvested on filtermats which finally were counted in a scintillation counter. All assays were performed in triplicates in 200 ml final volume in round bottom 96 well microtiter plates

Proliferation assay

Induction of T helper cells was assayed by direct proliferation of cells isolated from spleens or lymph nodes of mice immunized with *S. typhimurium* *aroA* strains, harboring the eukaryotic expression vector pCMV β , pCMVhly or pCMVActA or the prokaryotic β - gal expression vector pAH97. Proliferation of T cells were directly analysed by 3 H-thymidine incorporation.

Briefly, 2×10^5 T cells were co-cultured with 1×10^5 irradiated syngeneic spleen cells together with either 0.5 $\mu\text{g}/\text{ml}$ purified LLO, 1 $\mu\text{g}/\text{ml}$ Act-A or 1 $\mu\text{g}/\text{ml}$ β - gal. After 48 h of incubation at 37°C, 1mCi ^3H -thymidine was added to each culture and after further 18 h of incubation, cells were harvested on filter mats and incorporation was counted in a scintillation counter. All experiments were performed in triplicates in 200 ml final volume in flat bottom 96 well microtiter plates.

ELISA

To evaluate the levels of immunoglobulins against LLO, Act-A and β - gal in serum specimens, 96-well ELISA plates (Maxisorp, Nunc) were coated with 0.5 $\mu\text{g}/\text{ml}$ purified protein overnight at 4°C. Plates were washed three times with PBS/0.05% Tween 20 and then blocked with 3% BSA-PBS for 2 h at 37°C. Following two washes with PBS/0.05% Tween 20, serum samples of a 1:100 dilution were added to individual wells and incubated for 2-3 h at 37°C. Plates were washed as above and biotinylated goat anti-mouse Ig (Dianova, Hamburg, Germany) in 1% BSA-PBS was added to each well and incubated for 1 h at 37°C. After three washes with PBS/0.05% Tween 20, horseradish peroxidase conjugated streptavidin (Dianova, Hamburg, Germany) in 1% BSA-PBS was added to each well and incubated for 1 h at 37°C. Plates were washed as above, developed with *o*-Phenylenediamine as substrate and measured in an ELISA reader at 490 nm. For antigen specific IgG subclasses determination, peroxidase conjugated goat anti mouse IgG1, IgG2a, IgG2b and IgG3 (Caltag laboratories, CA, USA) were used.

Detection of β -gal activity

Expression of β -gal in host cells was monitored by incubating the fixed cells with the indicator substrate X-gal. Briefly, isolated peritoneal macrophages were allowed to adhere for a couple of hours at 37°C in 24 well-plate in antibiotic free medium. After removing the non-adherent cells and washing with antibiotic free medium, *S. typhimurium aroA*, harboring the eukaryotic expression vector pCMV β were added to the cells at a MOI of 10 and incubated at 37°C for 15-30 min. Cells were washed again and bacteria remaining extracellular were killed by addition of medium containing 50 $\mu\text{g}/\text{ml}$ gentamicin. Following 4 h of incubation at 37°C, 10 $\mu\text{g}/\text{ml}$ of tetracycline was added to some of the cultures to block the intracellular bacterial multiplication and incubation was continued for further 24 h. This second antibiotic step was later found to be unnecessary because strains harboring the *aroA* mutation survive only for brief periods of time in these cells (data not shown). After 2-3 washes with PBS, cells were fixed with

acetone/methanol (1:1 v/v) and freshly prepared X-gal substrate (5 mM potassium ferrocynide, 5 mM potassium ferricyanide, 2 mM MgCl₂ and 100 mg/ml X-gal in PBS) was added. After overnight incubation at 37°C β- gal expressing cells were detected by light microscopy. Quantitation of β-gal enzymatic activity in recombinant bacteria was determined according to standard procedures (Sambrook et al., 1982). Background (4U) was subtracted from the experimental values.

RNA isolation and RT-PCR

In order to test for expression of β- gal transferred into the eukaryotic host cells via *Salmonella*, the mRNA was probed for the presence of splice products derived from the splice donor and acceptors of the expression plasmid. To this end, PECs were infected *in vitro* at a MOI of 10 with *S. typhimurium aroA* harboring the eukaryotic expression vector pCMVβ and RNA was extracted as described (Chomczynski and Sacchi, 1987). RT-PCR of isolated RNA was performed. Briefly, 10 µg of isolated total cellular RNA was resuspended in 20 µl of DEPC-H₂O and incubated for 5 min at 70 °C with 10 µl of buffer containing 6 µl of reverse transcriptase buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂); 0,4 mM dNTPs; 0,05 U random hexamers (Pharmacia, Uppsala, Sweden); and 1 mM DTT. Samples were spun down for 2 min at 15,000 rpm and 40 U RNAsin ribonuclease inhibitor (Promega) together with 200 U Superscript reverse transcriptase (Gibco, BRL) were added. RNA was reverse transcribed for 45 min at 37°C and the reaction was stopped by heating the samples at 95°C for 1 min followed by a short incubation on ice. Subsequently 500 ng of cDNA product was amplified by PCR in a final volume of 50 µl containing 0,2 mM dNTP, 20 mM DTT, 3 µM of each of the 5' and 3' primer, 5 µl of 10x PCR buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 1% gelatine, 1,5 mM MgCl₂, 1% Triton X-100) and 5 U AmpliTaq-DNA-polymerase (Perkin Elmer). PCR was performed with an initial denaturation step of 10 min at 85°C followed by 35 cycles of 20 sec denaturation at 95°C, 30 sec annealing at 60°C and 30 sec extension at 72°C. The amplification products were visualized under an UV lamp after electrophoresis of a 15 µl aliquot of the reaction mixture on a 2% (w/v) agarose gel containing 0,5 µg/ml of ethidium bromide. The primer pair was designed in such a way that the presence of splice products should be indicated by a 190 bp and/or a 125 bp fragment. The identity of the presumable splice product was confirmed by sequencing the fragments after isolation on a preparative agarose gel. The primer pair used for amplification and sequencing - SV40 forward: 5'-GGATCCGGTACTCGAGGAAC-3', SV40 reverse : 5'-GCTTTAGCAGGCTTTCG-3'.

Immunoprecipitations

Biosynthetic labeling of proteins in the presence of tetracycline followed by immunoprecipitation should only reveal protein expression by eukaryotic host cells. Therefore, 5×10^5 adherent PECs were infected for 30 min at 37°C with ca. 5×10^6 *S. typhimurium* *aroA* harboring the eukaryotic expression vector pCMV β in antibiotic free medium. After a thorough wash and further 4 h of incubation at 37°C, medium was supplemented with antibiotics or not and left at 37°C for various periods of time before biosynthetic labeling. After two washes in PBS and 30 min starvation in methionine-free medium, cells were pulsed with 100 μ Ci [35 S]methionine for 2 h. Then cells were carefully washed and lysed in 0.5 ml ice cold lysis buffer (0.5% NP-40, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 1 mM PMSF). After 45 min on ice, cells were centrifuged to remove nuclei and cell debris, and incubated at 4°C with 4 μ g of anti- β -gal antibodies (Promega) for 30 min. Immune complexes were precipitated with protein A sepharose in 0.5% NP-40, 50 mM Tris-HCl pH 7.4, 5 mM MgCl₂ and 0.5mM NaCl washed several times with the same buffer and analysed on 8% SDS-PAGE followed by fluorography. Into some samples a 100-fold excess of β - gal protein was added before addition of anti- β -gal antibodies to determine the specificity of the precipitation.

The disclosure comprises also that of EP 97 106 503.2.

13

References :

Aggrawal, A., Kumar, S., Jaffe, R., Hone, D., Gross, M., and Sadoff, J. (1990). Oral *Salmonella*: malaria circumsporozoite recombinants induce specific CD8⁺ cytotoxic T cells. *J. Exp. Med.* 172: 1083-1090

Brooks, A. G., and McCluskey, J. (1993). Class II-restricted presentation of hen egg lysozyme determinant derived from endogenous antigen sequestered in the cytoplasm or endoplasmic reticulum of the antigen presenting cells. *J. Immunol.* 150:3690-3697

Brown, A., Hormaeche, C. E., Demarco de Hormaeche, R., Winther, M., Dougan, G., Maskell, D. J., and Stocker, A. D. (1987). An attenuated *aroA* *Salmonella typhimurium* vaccine elicits humoral and cellular immunity to cloned b-galactocidase in mice. *J. Infect. Dis.* 155:86-91

Chakraborty, T., Leimeister-Wächter, M., Domann E., Hartl, M., Goebel, W., Nichterlein, T., and Notermanns, S. (1992). Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. *J. Bacteriol.* 174:568-574.

Chatfield, S. N., Dougan, G., and Roberts, M. (1994). Progress in the development of multivalent oral vaccines based on live attenuated *Salmonella*. In E. Kurstak (ed.), *Modern vaccinology*. Plenum Medical, New York. p.55-86

Chomczynski, P., and N. Sacchi. (1987). Single step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156-159.

Condon, C., Watkins, S. C., Celluzzi, C. M., Thompson, K., and Faldo, Jr. L. D. (1996). DNA-based immunization by in vivo transfection of dendritic cells. *Nature med.* 2:1122-1128

Courvalin, P., Goussard, S., and Courvalin, C. G. (1995). Gene transfer from bacteria to mammalian cells. *C. R. Acad. Sci. Paris.* 318:1207-1212

Darji, A., Chakraborty, T., Wehland, J., and Weiss, S. (1995a). Listeriolysin generates a route for the presentation of exogenous antigens by major histocompatibility complex class I. *Eur. J.*

24

Immunol. 25:2967-2971

Darji, A., Chakraborty, T., Niebur, N., Tsionis, N., Wehland, J., and Weiss, S. (1995b). Hyperexpression of listeriolysin in the nonpathogenic species *Listeria innocua* and high yield purification. J. Biotechnol. 43:205-212

Darji, A., Chakraborty, T., Wehland, J., and Weiss, S. (1997). TAP-dependent major histocompatibility complex class I presentation of soluble proteins using listeriolysin. Eur. J. Immunol. 27:1353-1359

Domann E., Wehland J., Rohde M., Pistor S., Hartl M., Goebel W., Leimeister-Wächter M., Wuenscher M., and Chakraborty T. (1992). A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. EMBO J. 11:1981-1990.

Donnelly, J. J., Ulmer, J. B., Shiver, J. W., and Liu, M. A. (1997). DNA vaccines. Annu. rev. Immunol. 15:617-648

Germanier, R. and Fürer, E. (1975). Isolation and characterization of *Gal E* mutant Ty 21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine. J. Infect. Dis. 131: 553-558

Fairweather, N. F., Chatfield, N., Charles, I. G., Roberts, M., Lipscomb, M., Jing Li, J., Strugnell, D., Comerford, S., Tite, J., and Dougan, G. (1990). Use of live attenuated bacteria to stimulate immunity. Res. Microbiol. 141:769-773

Fox, B.C. (1997). Safety and efficacy of an avirulent live *Salmonella choleraesuis* vaccine for protection of calves against S dublin infection. Am. J. Vet. Res. 58:265-271

Flynn, J. L., Weiss, W. R., Norris, K. A., Seifert, H. S., Kumar, S., and So, M. (1990). Generation of cytotoxic T-lymphocyte response using *Salmonella* antigen-delivery system. Mol. Microbiol. 4:2111-2118.

25

Geissler, M., Geisen, A., Tokushige, K., and Wands, J. R. (1997). Enhancement of cellular and humoral immune response to Hepatitis C virus core protein using DNA-based vaccines augmented with cytokine-expressing plasmids. *J. Immunol.* 158:1231-1237

Guzmán, C. A., Brownlie, R. M., Kadurugamuwa, J., Walker, M. J., and Timmis, K. N. (1991). Antibody responses in the lung of mice following oral immunization with *Salmonella typhimurium aroA* and invasive *Escherichia coli* strains expressing the filamentous hemagglutinin of *Bordetella pertussis*. *Infect. Immun.* 59:4391-4397

Hanahan, D. (1983). Studies in transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:577-580.

Harty, J. T. and Bevan, M. J. (1992). CD8⁺ T cells specific for a single nonamer epitope of *Listeria monocytogenes* are protective *in vivo*. *J. Exp. Med.* 175:1531-1538

Hassan, J.O. (1996). Effect of vaccination of hens with an avirulent strain of *Salmonella typhimurium* on immunity of progeny challenged with wild-Type Salmonella strains. *Infect. Immun.* 64:938-944

Hess, J., Gentschev, I., Miko, D., Welzel, M., Ladel, C., Goebel, W., and Kaufmann, S. H. E. (1996). Superior efficacy of secreted over somatic antigen display in recombinant *Salmonella* vaccine induced protection. *Proc. Natl. Acad. Sci. USA* 93:1458-1461

Holtel, A., Timmis, K. N., and Ramos, J. L. (1992). Upstream binding sequences of the XyIR activator protein and integration host factor in the *xyIS* gene promoter region of *Pseudomonas* TOL plasmid. *Nucleic Acids Res.* 20:1755-1762

Hunter, S. J., F. R. Carbone., and R. A. Strugnell. (1993). *Salmonella typhimurium aroA-aroD-* mutants expressing a foreign recombinant protein induce specific major histocompatibility complex class I-restricted cytotoxic T lymphocytes in mice. *Infect. Immun.* 61:5374-5380.

Kim, J. J., Ayyavvo, V., Bagarazzi, M. L., Chattergoon, M. A., Dang, K., Wang, B., Boyer, J.

26

D., and Weiner, D. B. (1997). *In vivo* engineering of a cellular immune response by coadministration of IL-12 expression vector with a DNA immunogen. *J. Immunol.* 158:816-826

Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretsky, G. A., and Klinman D. M. (1995). CpG motif in bacterial DNA trigger direct B-cell activation. *Nature* 374:546-548

Matzinger, P. (1991). The JAM test. A simple assay for DNA fragmentation and cell death. *J. Immunol. Methods*. 145: 185-192

Mengaud, J., Vicente M-F., Chenevert J., Pereira J. M., Geoffroy C., Gicquel-Sanzey B., Baquero F., Perez-Diaz J-C., and Cossart P. (1988). Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. *Infect. Immun.* 56:766-772.

Messina, J. P., Gilkeson, G. S., and Pisetsky, D. S. (1991). Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. *J. Immunol.* 147:1759-1754

Molina, C. N., and Parker, C. D. (1990). Murine antibody response to oral infection with live *aroA* recombinant *Salmonella dublin* vaccine strains expressing filamentous hemagglutinin antigen from *Bordetella pertussis*. *Infect. Immun.* 58:2523-2528

Moore, M. W., Carbone, F. R., and Bevan, M. J. (1988). Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell*. 54:777-785

Mosmann, T. R., and Coffman, R. L. (1989). TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol.* 7:145-173

Neutra, M. R., Pringault, E., and Kraehenbuhl J-P. (1996). Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annu. Rev. Immunol.* 14: 275-300

Newton, S. M. C, Jacob, C. O., and Stocker B. A. D. (1989). Immune response to cholera toxin

27

epitope inserted in *Salmonella* flagellin. *Science* 244:70-72

Norbury, C. C., Helwett, L. J., Prescott, A. R., Shastri, N., and Watts, C. (1995). Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity* 3:783-791

Nuchtern, J. G., Bonifacino, J. S., Biddison, W. E., and Klausner, R. D. (1989). Brefeldin A implicates egrees from endoplasmic reticulum in class I restricted antigen presentation. *Nature (London)*. 361:359-362

O'Callaghan, D., and Charbit A. (1990). High efficiency transformation of *Salmonella typhimurium* and *Salmonella typhi* by electroporation. *Mol. Gen. Genet.* 223:156-158.

Pamer, E. G., Harty, J. T., and Bevan, M. J. (1991). Precise prediction of a dominant class I MHC-restricted epitope of *Listeria monocytogenes*. *Nature (London)*. 353:852-855

Rammensee, H. G., Schild, H., and Theopold, U. (1989). Protein specific cytotoxic T lymphocytes recognition of transfectants expressing intracellular, membrane-associated or secreted forms of beta-galactocidase. *Immunogenetics* 30:296-301

Reis de Sousa, C., and Germain, R. N. (1995). Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. *J. Exp. Med.* 182:841-851

Roberts, M., Chatfield, S. N., and Dougan, G. (1994). *Salmonella* as carriers of heterologous antigens. In D. T. O'Hagan (ed.), *Novel delivery system for oral vaccines*. CRC Press, Inc., Boca Raton, Fla. p.27-58

Sambrook, J., Fritsch E. F., and Maniatis T. (1982). *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Siebers, A., and Finlay, B. B. (1996). M cells and pathogenesis of mucosal and systemic

infections. *Trends Microbiol.* 4:22-29

Sher, A., and Coffman, R. L. (1992). Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Ann. Rev. Immunol.* 10:385-409

Sizemore, D. R., Branstrom, A. A., and Sadoff, J. C. (1995). Attenuated *Shigella* as a DNA delivery vehicle for DNA-mediated immunization. *Science* 270:299-302

Staats, H. F., Jackson, R. J., Marinaro, M., Takahashi, I., Kiyono, H., and McGhee, R. J. (1994). Mucosal immunity to infection with implications for vaccine development. *Curr. Opin. Immunol.* 6:572-583

Steinbach, G. (1996). Influence of prior infection on the dynamics of bacterial counts in calves experimentally infected with *Salmonella dublin*. *Vet. Microbiol.* 48:199-206

Tian, J., Clare-Salzler, M., Herschenfeld, A., Middleton, B., Newman, D., Mueller, R., Arita, S., Evans, C., Atkinson, M. A., Mullen, Y., Sarvetnick, N., Tobin, A. J., Lehmann, P. V., and Kaufman, D. L. (1996). Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nature Med.* 2:1348-1353

Turner, S. J., Carbone, F. R., and Strugnell, R. A. (1993). *Salmonella typhimurium aroA aroD* mutants expressing a foreign recombinant protein induce specific major histocompatibility complex class I-restricted cytotoxic T lymphocytes in mice. *Infect. Immun.* 61:5374-5380

Xiang, Z., and Ertl, H. C. J. (1995). Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity* 2:129-135

Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992). Unique palindromic sequences in synthetic oligonucleotides are required to induce TNF and augment TNF-mediated natural killer activity. *J. Immunol.* 148:4072-4076

Zhu, N., Liggitt, D., Liu, Y., and Debs R. (1993). Systemic gene expression after intravenous

29

DNA delivery into adult mice. Science 261:209-211

1. Attenuated *Salmonella* strain carrying an eucaryotic expression vector for the expression of a heterologous gene or gene fragment or an autologous gene or gene fragment comprised by the vector within an open reading frame, wherein the attenuation is adjusted to a vaccination of vertebrates including humans.
2. *Salmonella* strain according to claim 1, wherein the strain is a *S. typhimurium* strain, especially *S. typhimurium* aroA SL 7207 or *S. typhimurium* LT2 and preferably aroA544 (ATCC 33275).
3. *Salmonella* strain according to claim 1, wherein the strain is a *S. typhi* strain, especially *S. typhi* Ty21a.
4. *Salmonella* strain according to any of the preceding claims, wherein the eucaryotic expression vector is or can be derived from the known plasmid pCMV β which comprises
 - the structural gene of β -galactosidase (β -gal)

- under the control of the human CMV immediate early promotor comprised by the plasmid pCMV β per se and includes
- a splice donor,
- two splice acceptor sites in between the promotor and the β -galactosidase gene, and facultatively
- the polyadenylation site of SV40.

5. *Salmonella* strain according to any of the preceding claims, characterized by a heterologous gene or an autologous gene coding for a protein and especially an immunogenic protein or protective antigen.

6. *Salmonella* strain according to any of the preceding claims wherein the heterologous gene is selected from the group consisting of

- the *Escherichia coli*- β -galactosidase gene (*lacZ*-gene),
- a non-hemolytic truncated variant of the *Listeria monocytogenes*-listerio lysin gene (*hly* gene) and
- a truncated variant of the *Listeria monocytogenes*-*actA* gene (*actA* gene).

7. Vaccine for oral and/or nasal and/or mucosal gene delivery to vertebrates including humans, wherein the vaccine comprises a *Salmonella* strain according to any of the preceding claims.

8. Use of a *Salmonella* strain according to any of the preceding claims or of a vaccine according to claim 7 for expression screening of heterologous genomic DNA libraries or genomic cDNA libraries by DNA vaccination in vertebrates including humans.

9. Process for the recovery of

- (i) an attenuated *Salmonella* strain carrying an eucaryotic expression vector for the expression of a heterologous gene or gene fragment or an autologous gene or gene fragment comprised by the vector within an open reading frame, wherein the

attenuation is adjusted to a vaccination of vertebrates including humans; or

(ii) a vaccine for oral and/or nasal gene delivery to vertebrates including humans, wherein the vaccine comprises a *Salmonella* strain according to (i) or

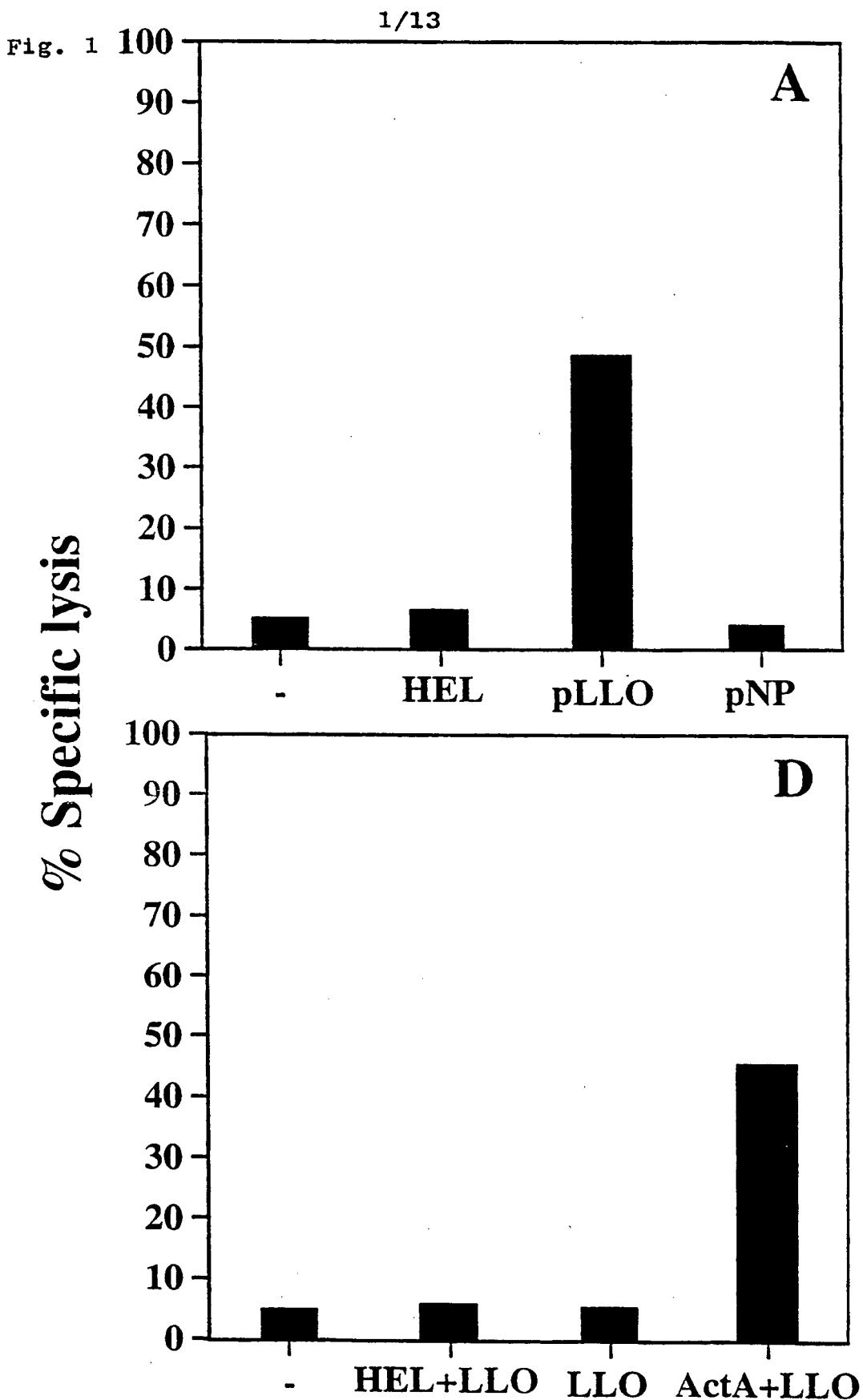
(iii) an immunogenic protein or protective antigen as expression product of an eucaryotic expression vector according to (i), characterized by

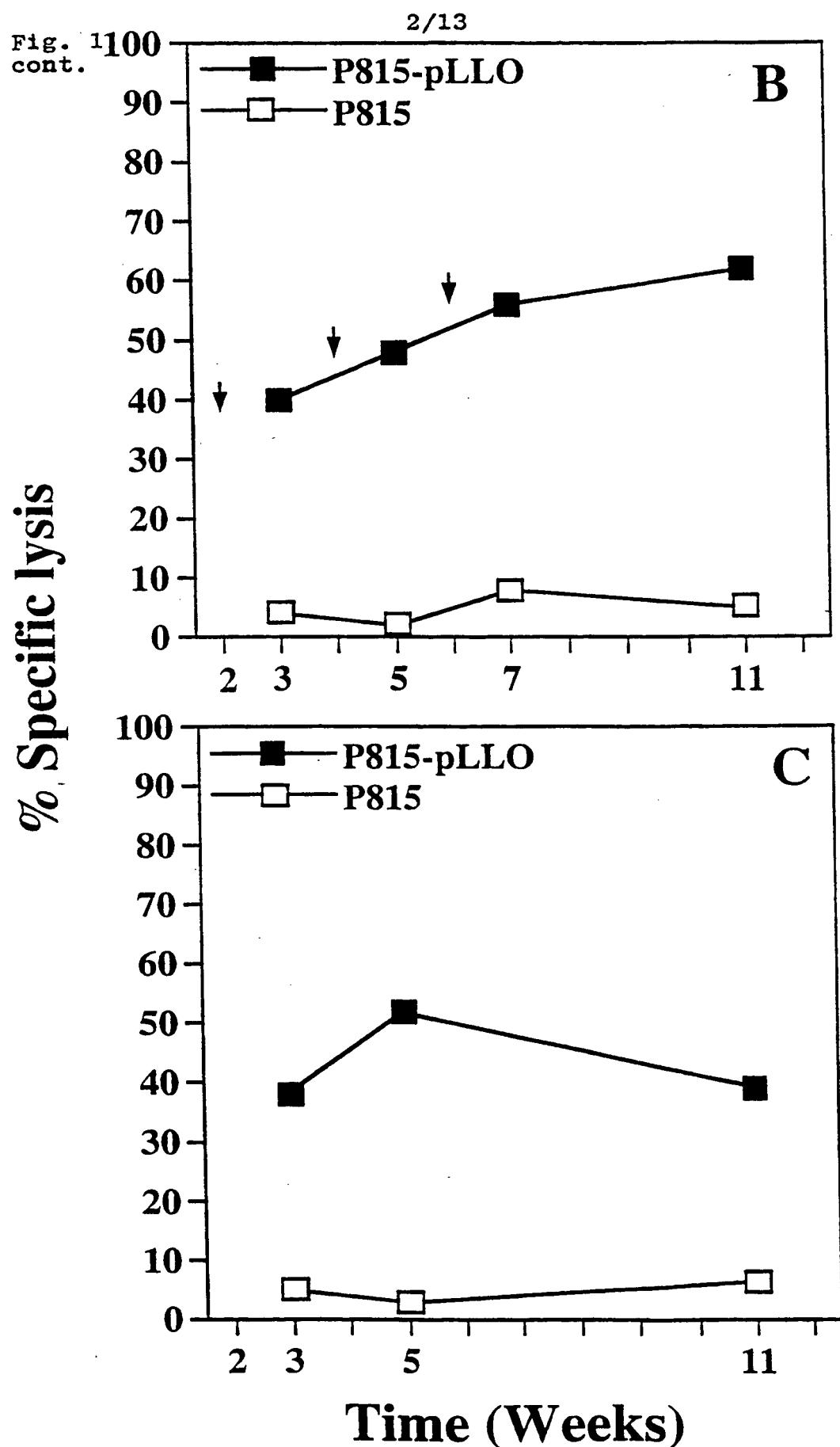
(a) using genetic information from a heterologous or autologous DNA or cDNA library as gene fragment or gene to be expressed by an eucaryotic expression vector carried by an attenuated *Salmonella* strain, wherein the attenuation is adjusted to a vaccination of vertebrates including humans,

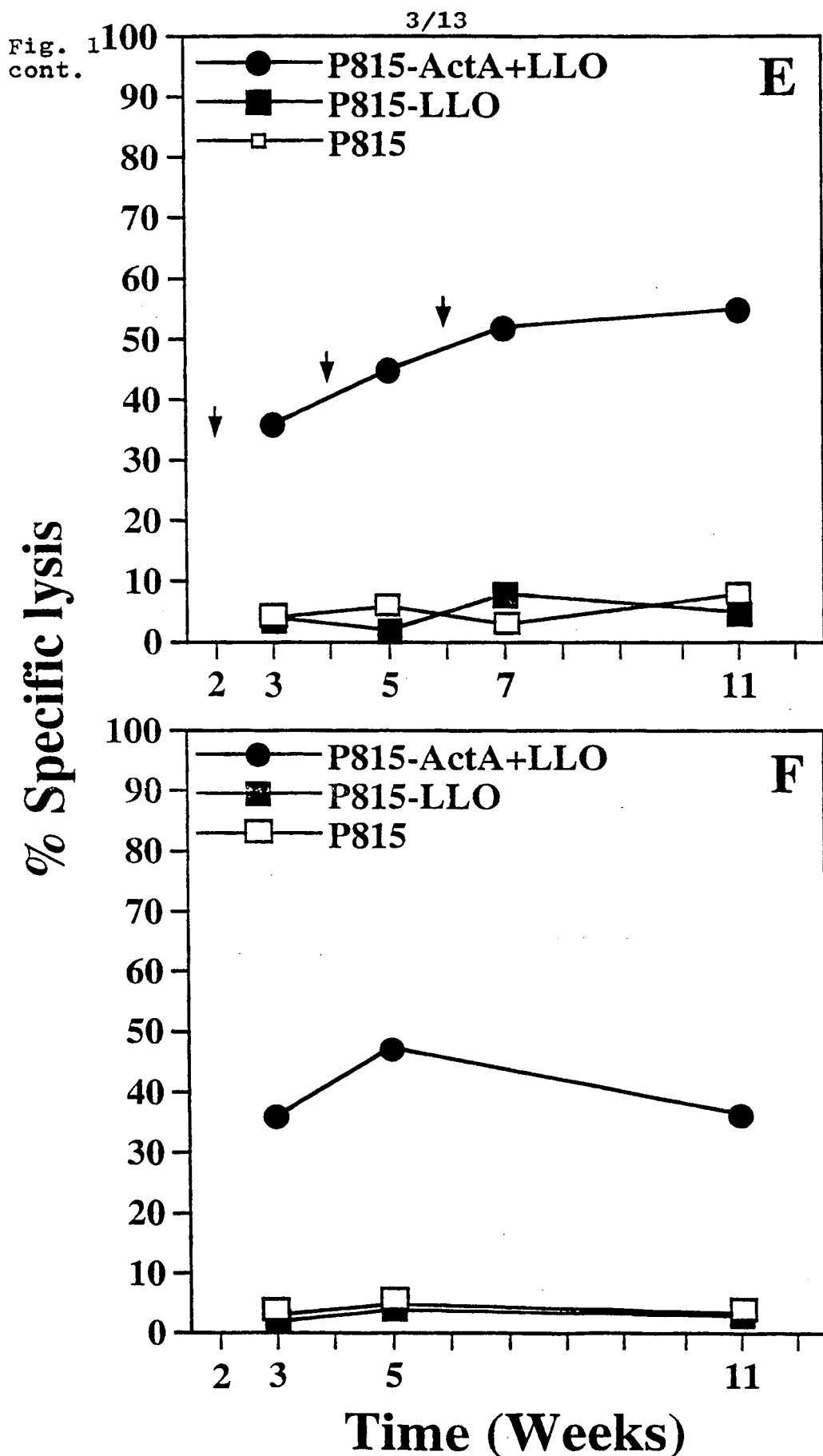
(b) carrying out a DNA vaccination by means of the attenuated *Salmonella* strain according to (a) in a vertebrate or human being,

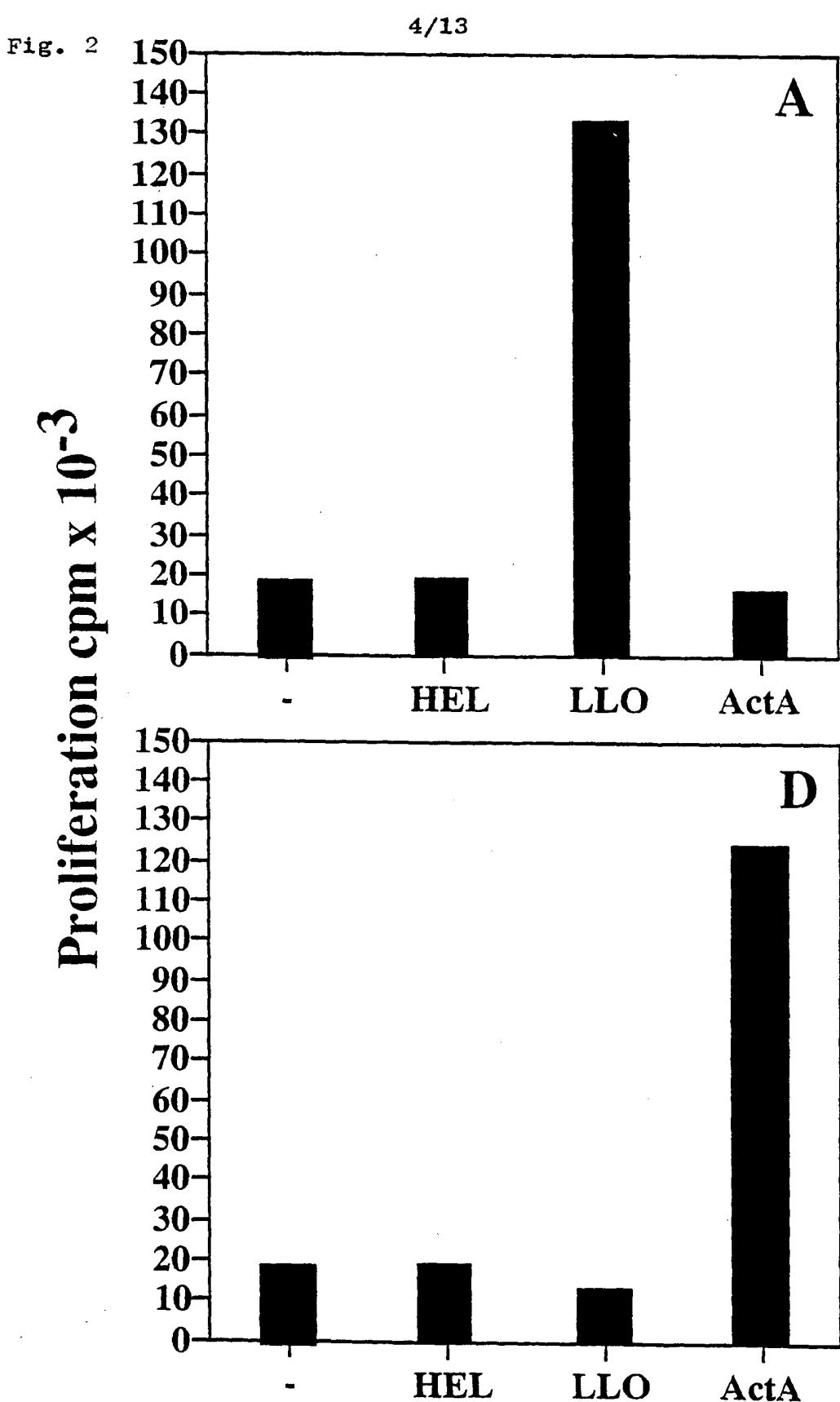
(c) carrying out an expression screening for an expression product of a gene or gene fragment according to (a) providing an immune response

(d) and recovering a *Salmonella* strain according to any of claims 1 to 6 or of a vaccine according to claim 7 or of an immunogenic protein or protective antigen providing an immune response in vertebrates including humans.









5/13

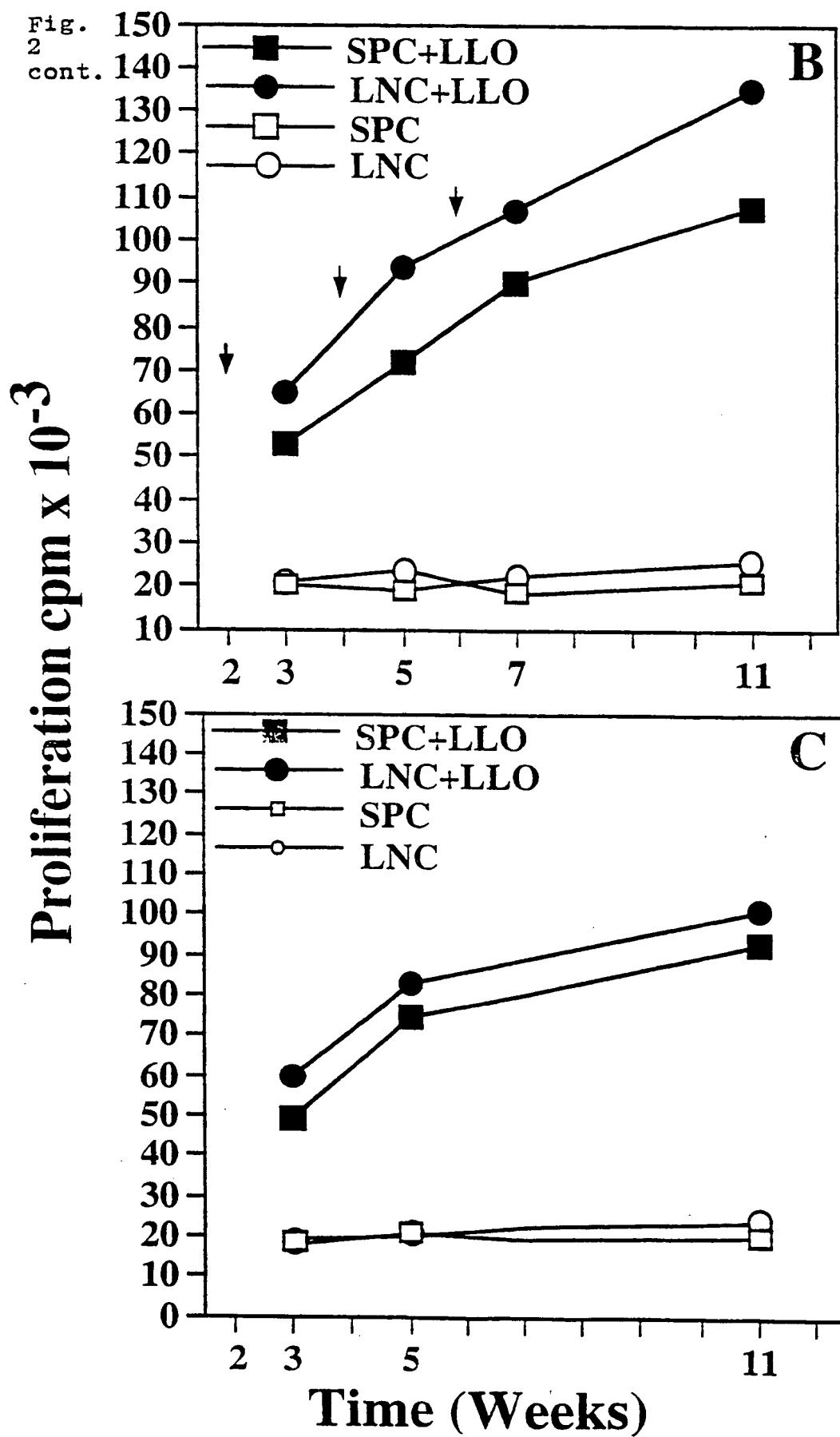
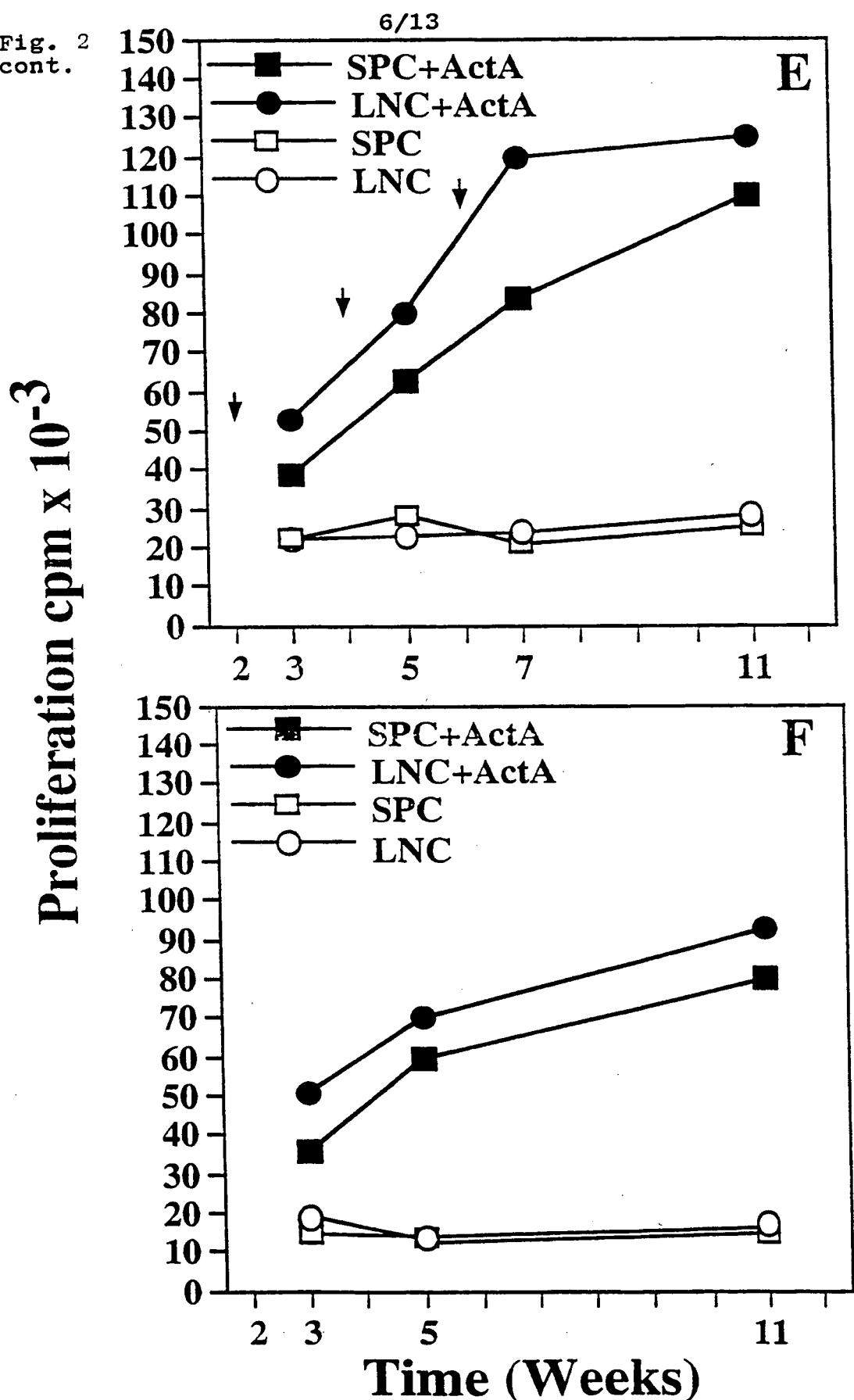
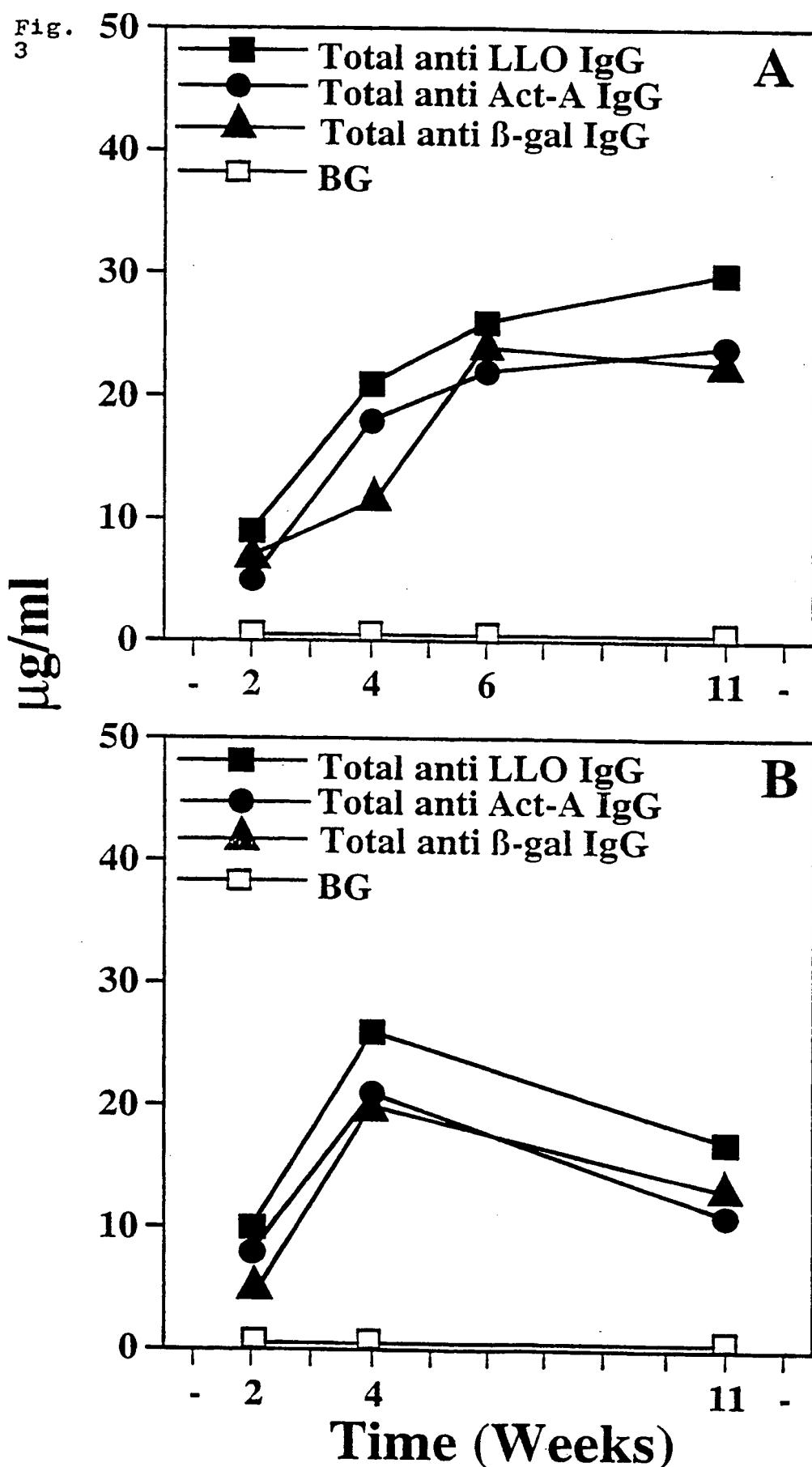
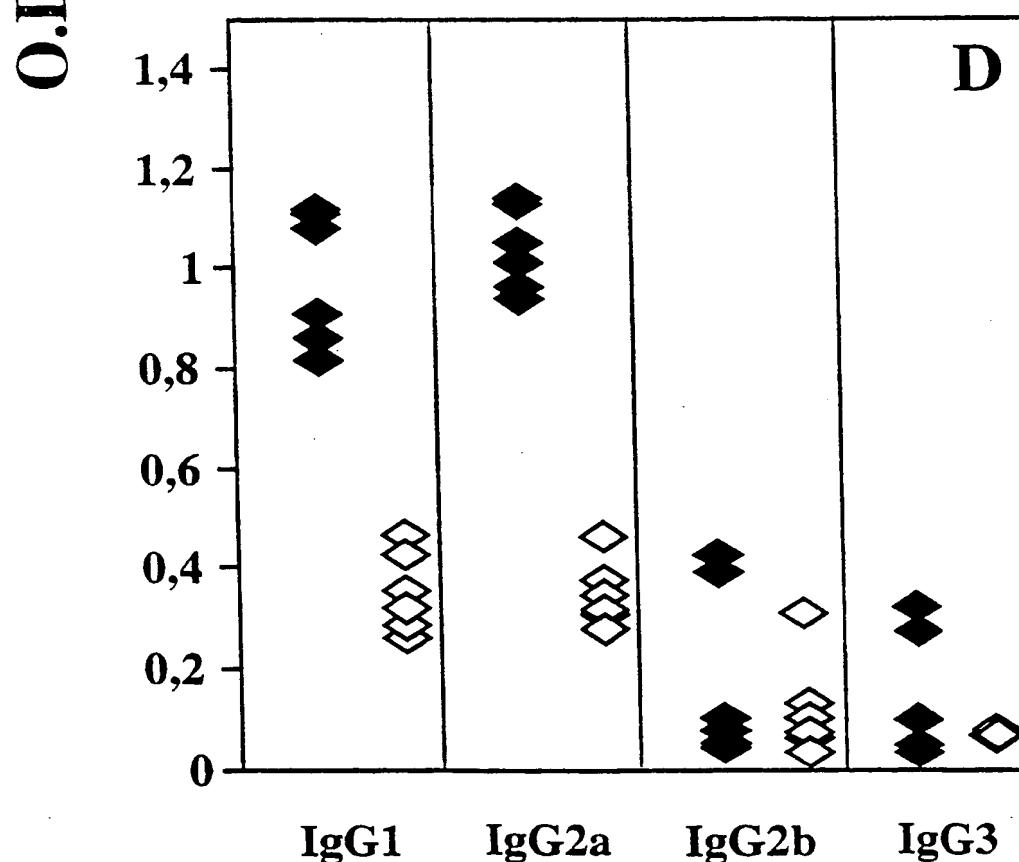
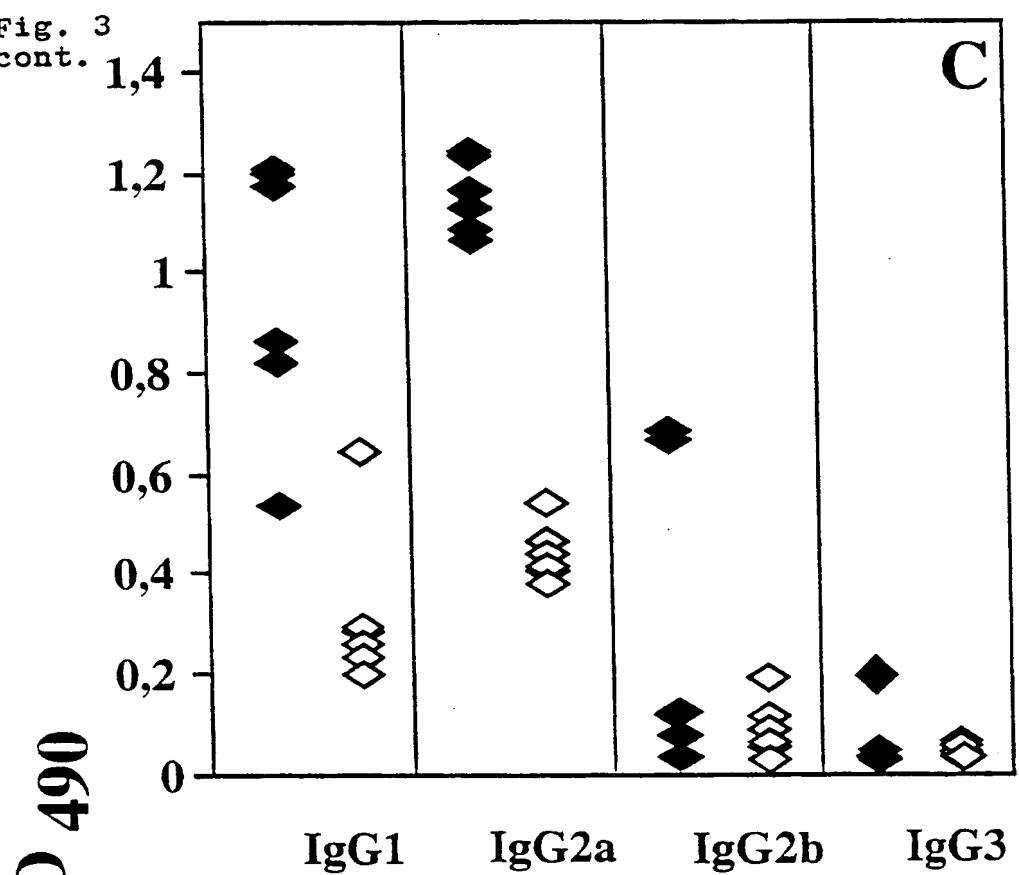


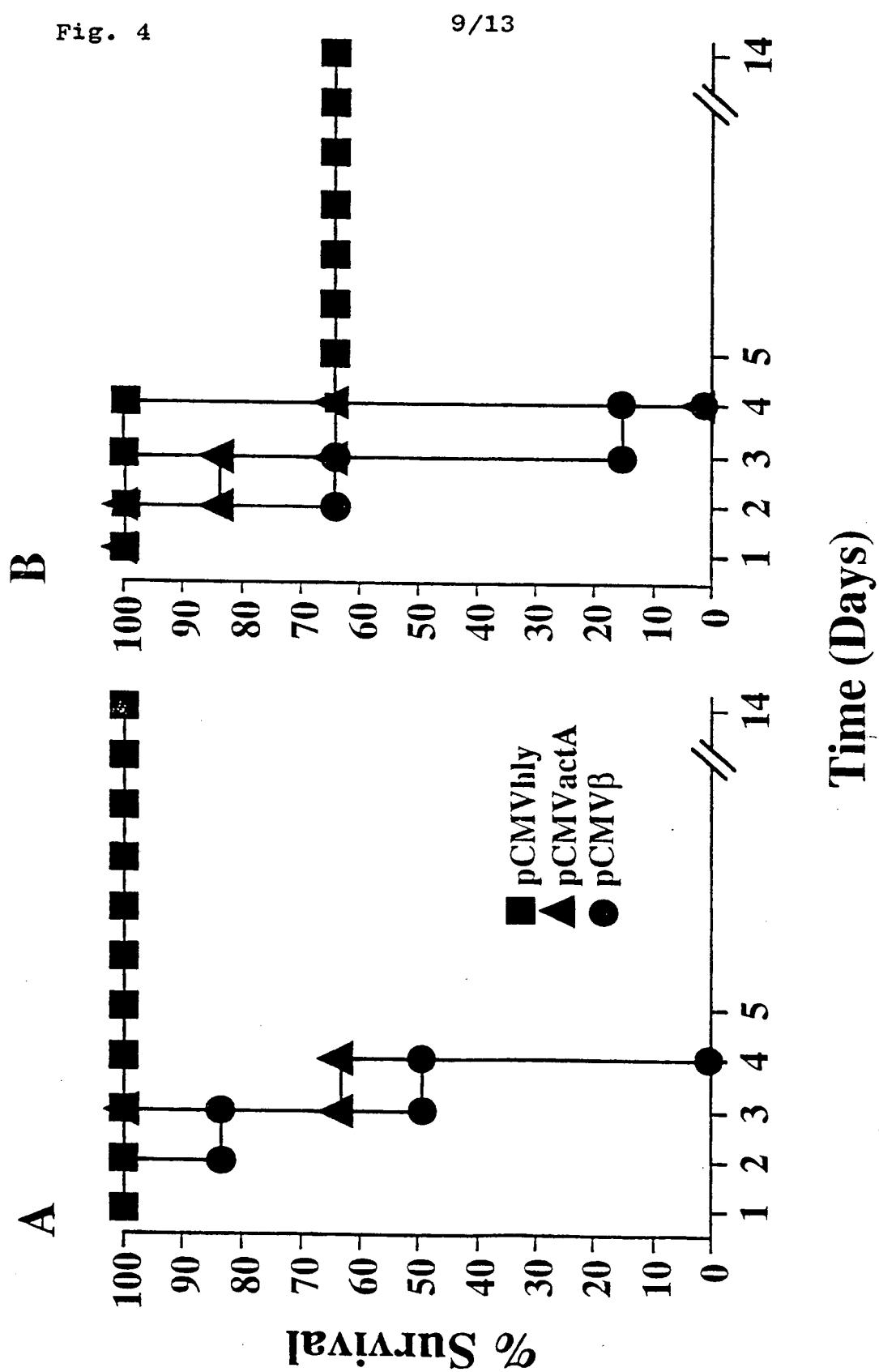
Fig. 2
cont.

7/13



8/13

Fig. 3
cont.



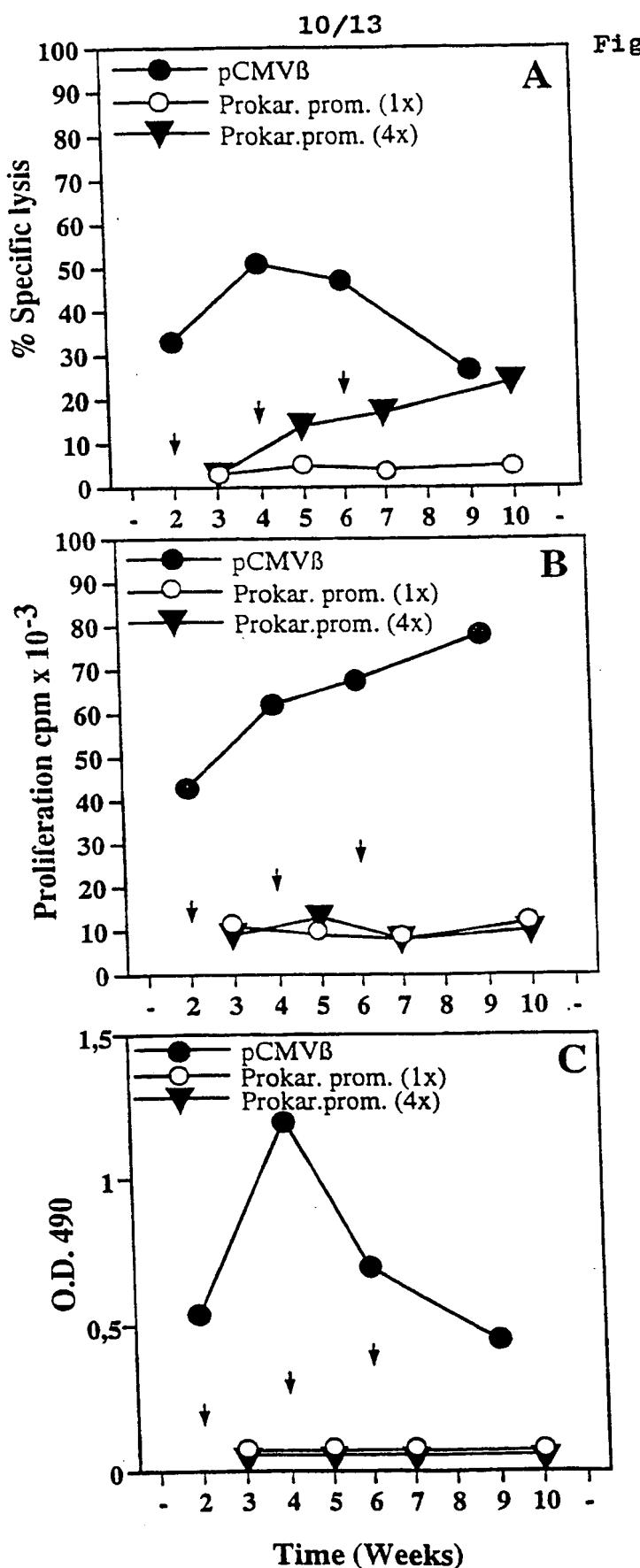
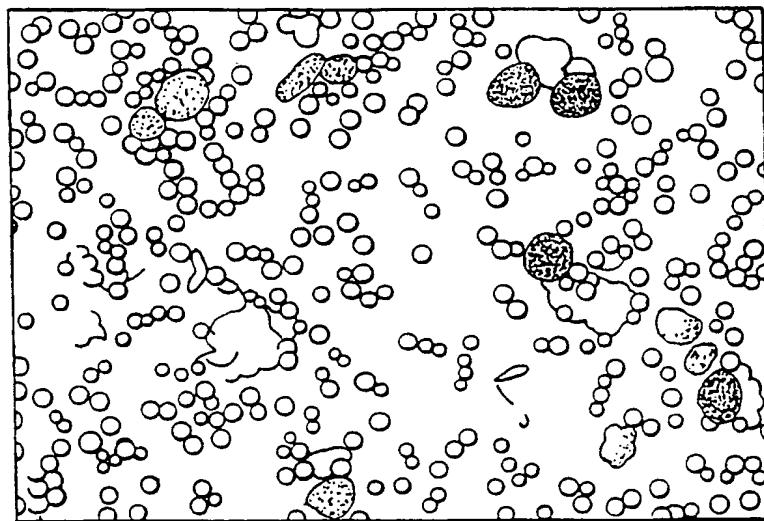


Fig. 5

11/13

Fig. 6



12/13

Fig. 7

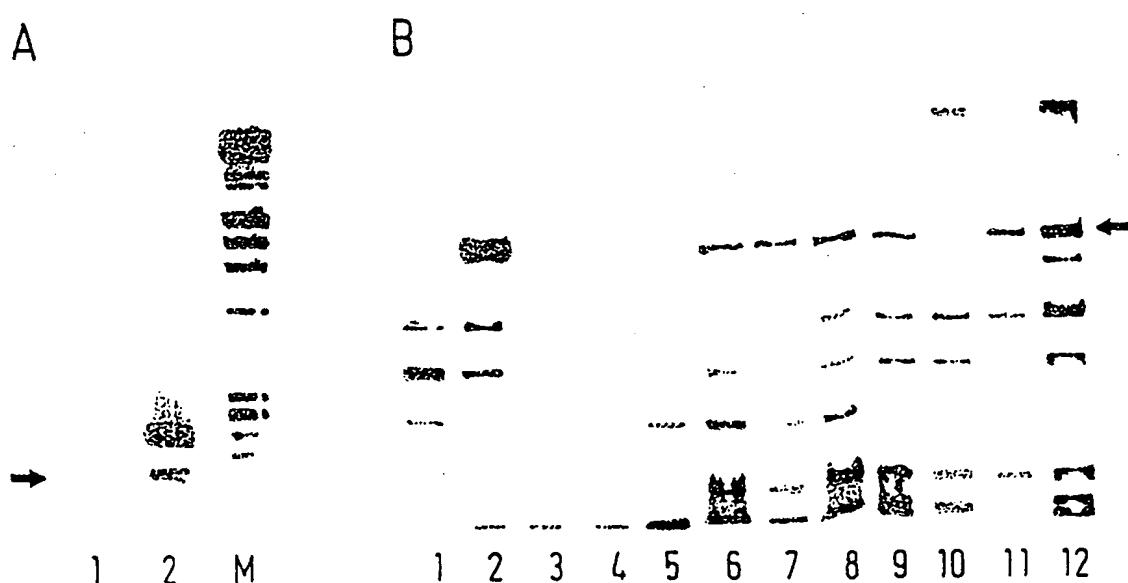
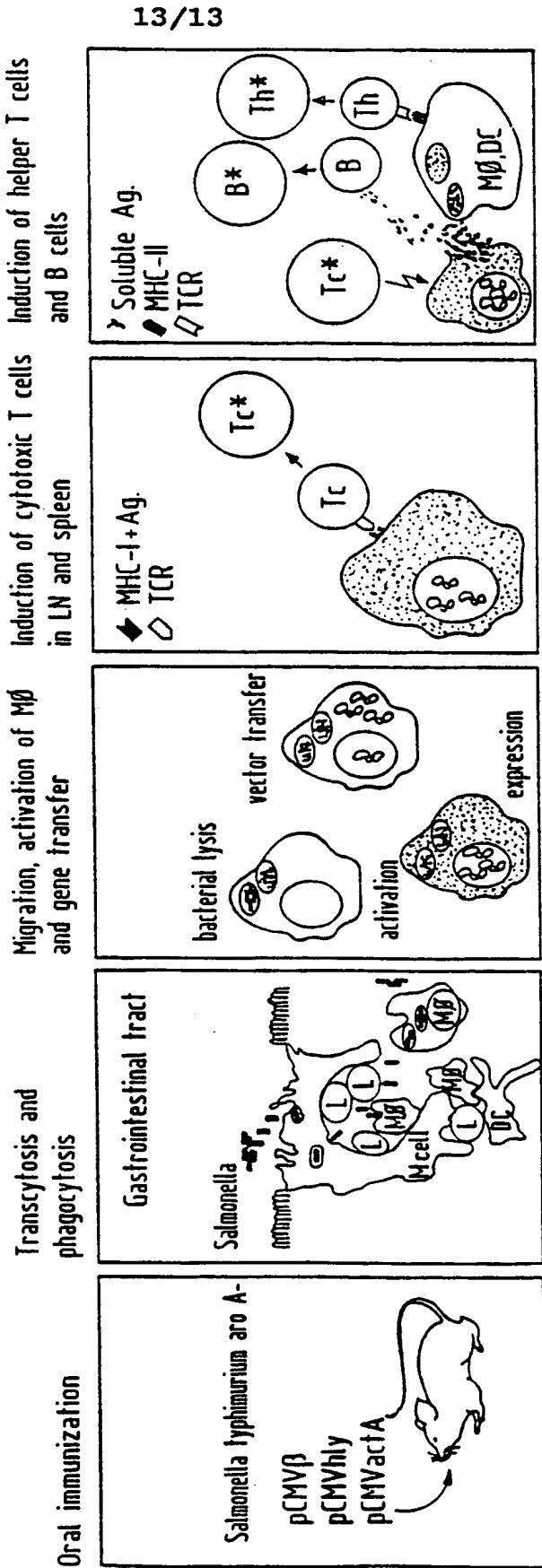


Fig. 8



INTERNATIONAL SEARCH REPORT

Application No
PCT/EP 97/06933

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/74 C07K14/195 A61K39/02 A61K39/112

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 08955 A (US ARMY ;BRANSTROM ARTHUR A (US); SIZEMORE DONATA R (US); SADOFF J) 13 March 1997 see the whole document ---	1-9
Y	COURVALIN ET AL.: "GENE TRANSFER FROM BACTERIA TO MAMMALIAN CELLS" COMPTEES RENDUES DE L'ACADEMIE DES SCIENCES, vol. 318, no. 12, 1995, pages 1207-1212, XP002071284 cited in the application see the whole document ---	1-9

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

14 July 1998

28/07/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Hagenmaier, S

INTERNATIONAL SEARCH REPORT

National Application No

PCT/EP 97/06933

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SIZEMORE ET AL.: "ATTENUATED SHIGELLA AS A DNA DELIVERY VEHICLE FOR DNA-MEDIATED IMMUNIZATION" SCIENCE, vol. 270, 1995, pages 299-302, XP002071287 cited in the application see the whole document ---	1-9
Y	TURNER ET AL.: "SALMONELLA TYPHIMURIUM aroA aroD MUTANTS EXPRESSING A FOREIGN RECOMBINANT PROTEIN INDUCE SPECIFIC MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I-RESTRICTED CYTOTOXIC T LYMPHOCYTES IN MICE" INFECTION AND IMMUNITY, vol. 61, no. 12, 1993, pages 5374-5380, XP002071285 see the whole document ---	1-9
Y	DOMANN ET AL.: "A NOVEL BACTERIAL VIRULENCE GENE IN LISTERIA MONOCYTOGENES REQUIRED FOR HOST CELL MICROFILAMENT INTERACTION WITH HOMOLOGY TO THE PROLINE-RICH REGION OF VINCULIN" EMBO J., vol. 11, no. 5, 1992, pages 1981-1990, XP002071288 cited in the application see the whole document ---	6
Y	MENGAUD E AL.: "EXPRESSION IN ESCHERICHIA COLI AND SEQUENCE ANALYSIS OF THE LISTERIOLYSIN O DETERMINANT OF LISTERIA MONOCYTOGENES" INFECTION AND IMMUNITY, vol. 56, no. 4, 1988, pages 766-772, XP002071289 cited in the application see the whole document ---	6
A	BROWN ET AL.: "AN ATTENUATED aroA SALMONELLA TYPHIMURIUM VACCINE ELICITS HUMORAL AND CELLULAR IMMUNITY TO CLONED BETA-GALACTOSIDASE IN MICE" J. INF. DISEASE, vol. 155, no. 1, 1987, pages 86-92, XP002071286 see the whole document ---	1-9
		-/-

INTERNATIONAL SEARCH REPORT

In Application No
PCT/EP 97/06933

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SIZEMORE ET AL.: "INTERACTION OF SALMONELLA TYPHI STRAINS WITH CULTURED HUMAN MONOCYTE-DERIVED MACROPHAGES" INFECTION AND IMMUNITY, vol. 65, no. 1, January 1997, pages 309-312, XP002071319 see the whole document -----	1-9
P, X	DARJI ET AL.: "ORAL SOMATIC TRANSGENE VACCINATION USING ATTENUATED S.TYPHIMURIUM" DARJI ET AL., vol. 91, December 1997, pages 765-775, XP002071290 see the whole document -----	1-9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 97/06933

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Although claim 9 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking(Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int

Application No

PCT/EP 97/06933

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9708955	A 13-03-1997	AU 7105996 A	27-03-1997